A STUDY OF FACTORS AFFECTING THE TOXICITY OF CHOLINESTERASE INHIBITORS

By

Peter Arnold Adie

1958

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ABSTRACT

The relationship between detoxication and toxicity has been examined with three cholinesterase inhibitors, namely, isopropyl methylphosphonofluoridate (sarin), ethyl N,N-dimethylphosphoramidocyanidate (tabun) and 0,0-diethyl S-ethyl-2-mercaptoethyl phosphorothiolate (isosystox). Sarin and tabun are both hydrolyzed enzymically in tissue but isosystox is not.

When plasma is subjected to denaturation by heat or by acid and alkali, the hydrolysis of both sarin and tabun is affected in a similar manner. A 1200-fold purification of the enzyme (sarinase) which hydrolyzes sarin produces a similar purification of the enzyme (tabunase) which hydrolyzes tabun. In addition, the distribution of sarinase in liver cell fractions follows closely that of tabunase. These findings suggest that sarinase and tabunase are identical. Other evidence pointing to the same conclusion is the close agreement between the activation energies (15,000 cal. with sarin and 14,600 cal. with tabun). Although the same enzyme hydrolyzes both sarin and tabun, different enzyme sources were found to exhibit different substrate specificities.

A calculation based on the Michaelis constant ($K_m = 7.0 \times 10^{-3}$ M sarin and 7.6×10^{-3} M tabun) indicated that plasma sarinase is able to destroy 50% of the injected sarin or tabun in less than one minute. Nevertheless, there appears to be no relationship between the amount of sarinase in plasma and the susceptibility of the animal to sarin. However, when sarin is injected intraperitoneally so that the major portion must pass through the liver before entering the general circulation, the liver sarinase detoxifies an appreciable portion of the injected dose

and a relationship between liver sarinase and susceptibility to sarin exists.

In the case of isosystox, oxidation followed by hydrolysis occurs and, in addition, a small amount of an injected dose is excreted in urine. However both of these mechanisms were shown to be relatively unimportant. The major portion of an injected dose of isosystox appears to combine with non-essential cholinesterase and other esterases in the body.

The high toxicity of sarin and tabun must be a result of the extremely fast reaction of these compounds with essential cholinesterase. The pseudomonomolecular rate constant for the inhibition of cholinesterase by sarin is 7×10^7 litre $\text{mole}^{-1}\text{min}^{-1}$ and by tabun is 1×10^7 litre mole^{-1} min^{-1}. A calculation of the half life of essential cholinesterase shows that it is only 0.01 minutes when a lethal dose of sarin is injected and 0.035 minutes when tabun is injected. In contrast, the half life of essential cholinesterase when isosystox is injected is 23 minutes. These findings are supported by the differences in the times taken to kill animals when lethal amounts of these compounds are injected.

The significance of the results obtained has been discussed in relation to the treatment of anticholinesterase poisoning.

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Peter Arnold Adie

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GENERAL INTRODUCTION

I The Chemical Mediators of Nerve Transmission

The idea that a chemical mediator was involved in the transmission of nerve impulses was evolved early in the twentieth century. Following the work of Hunt (92), Hunt and Taveau (93), Elliot (59), and Dixon and Hamill (53), Dale in 1914 (44), suggested acetylcholine as the chemical mediator for parasympathetic transmission. He also postulated that an enzyme removed the active ester from circulation and restored the original sensitivity. Loewi and his associates (61, 112, 113, 114, 117, 118) investigated the chemical mediation of nerve impulses and showed that the vagus nerve liberates a substance which is pharmacologically and physiologically identical with acetylcholine.

The transmission in sympathetic nerves was examined by Cannon and co-workers (33, 34, 35). They showed that a substance was released into the circulation by these nerves which could produce a sympathetic effect on distant organs. This substance was thought to be adrenaline (24, 115); however later work has revealed it to contain mainly noradrenaline (120, 149).

Dale, in 1933 (45), proposed the name "cholinergic" for fibres that liberate acetylcholine and "adrenergic" for fibres that liberate an adrenaline-like substance.

The role of acetylcholine as a chemical mediator in the transmission of impulses from nerve endings to effector cells has been confirmed by many workers (46, 56, 66). One of the more important experiments was carried out by Dale and Feldberg (47). They demonstrated the presence of

"vagal substance" (acetylcholine) in the eserinized perfusion fluid collected from the stomach of a dog when the vagus was stimulated. The purpose of the eserine was to inhibit the hydrolysis of the acetylcholine by cholinesterase (the enzyme postulated by Dale in 1914). In 1936, Dale, Feldberg and Vogt (48) showed that stimulation of motor nerves produced acetylcholine but stimulation of muscle did not. They inferred that acetylcholine was released at the motor end plates when nerve impulses arrived at the neuromyal junction. More recently Eccles and MacFarlane (58), Fatt and Katz (62) and Kuffler (108) have all presented data showing that acetylcholine is responsible for transmission at motor neuromyal end plates.

The mediator for synaptic transmission in autonomic ganglia was examined by Feldberg and Gaddum (65) and Feldberg and Vartiainen (67). They showed that acetylcholine is released in the ganglion when preganglionic fibres are stimulated. The interpretation of the role of this substance in central synaptic transmission has been difficult since acetylcholine has both a stimulating and depressing effect (63). Feldberg (64) obtained evidence suggesting that there is an alternation between cholinergic and non-cholinergic neurones in the brain and spinal cord. Koelle's work (102) on the histochemical localization of cholinesterase appears to support this theory.

Acetylcholine has been demonstrated in axons and was presumed to be connected with the passage of action potentials (27, 63, 74, 116, 152). Later work, however, has shown that it is possible to inhibit the cholinesterase of the axon and thus build up the acetylcholine concentration without affecting transmission along the axon (30, 41). Furthermore acetyl-

choline does not initiate impulses in the preganglionic fibres of the stellate ganglion (31) or prevent conduction of impulses (119). Nachmansohn (142) attributed these results to the impermeability of the axon. He also postulated that acetylcholine has a role in axonal transmission.

Although the importance of acetylcholine in the transmission of action potentials along the axon has not yet been proven, its importance at the synapses and motor end plates is indisputable. Equally important is the means by which acetylcholine is removed from its site of action. The next section deals with this mechanism.

II Cholinesterase

Acetylcholine has a depolarizing action on the cell membrane (148) and it is essential once an action potential has been initiated, that the membrane be repolarized. This may be achieved only if acetylcholine is removed or destroyed. As mentioned above, Dale suggested that an enzyme was responsible for the destruction of acetylcholine (44). LeHeux (110) and Abderhalden and Paffrath (1) found that extracts of pig gut could hydrolyze acetylcholine and this was confirmed with other tissue extracts by Loewi and Navatril in 1926 (118). The enzymic nature of the hydrolysis was proven in 1930 by Engelhart and Loewi (61) and Matthes (123).

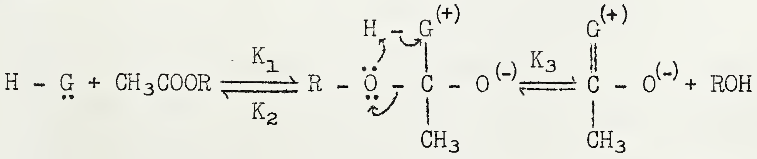
For some time the specificity of the esterase was in doubt (14, 57, 81, 156). In 1940, however, Alles and Hawes (13) showed that the cholinesterases from different sources had different characteristics. Richter and Croft in 1942 (151) reported that the cholinesterase of erythrocytes differed from that of serum in having an absolute specificity towards choline esters. Mendel and associates (126, 127, 128, 129, 161) confirmed

the existence of a "pseudo" or non-specific cholinesterase in some enzyme sources, e.g. serum, and of a "true" or specific cholinesterase in other enzyme preparations, e.g. erythrocytes and brain. Hawkins and Gunter (83), in 1946, gave evidence that "pseudo" cholinesterase plays no essential part in the hydrolysis of acetylcholine *in vivo*.

In 1951 Nachmansohn and Wilson (146), on the basis of previous work (16, 101, 144, 145, 162), defined "true" cholinesterase as having, (a) a high affinity for acetylcholine, (b) a well defined optimum of substrate concentration and (c) a decreased rate of hydrolysis with increased length of acyl chain.

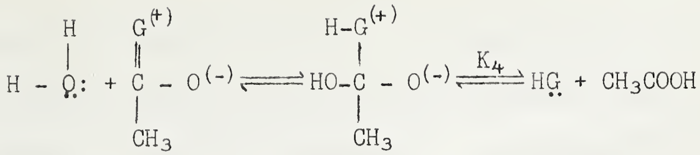
By 1954, work had been carried out on the mechanism of acetylcholine hydrolysis by cholinesterase from the point of view of intermolecular forces. Wilson (169) postulated two centres or "active" sites. One centre is an "anionic site" which reacts by coulombic forces with the cationic ammonium groups of choline esters. The other centre is an "esteratic site" which reacts with the ester group possibly by covalent bonding. From changes of esterase activity with pH, Bergmann (28) was able to calculate the pK values of the basic and acidic groups in the esteratic site of the enzyme (cf also 165, 166, 172).

The mechanism that Wilson suggested for acetylcholine hydrolysis is as follows:



$$H-\underset{\cdot\cdot}{G}+CH_3COOR \underset{K_2}{\overset{K_1}{\rightleftharpoons}} R-\underset{\cdot\cdot}{\overset{\cdot\cdot}{O}}-\underset{CH_3}{\overset{H-G^{(+)}}{C}}-O^{(-)} \overset{K_3}{\rightleftharpoons} \underset{CH_3}{\overset{G^{(+)}}{\overset{\|}{C}}}-O^{(-)}+ROH$$

A B



$$\mathrm{H-\underset{\cdot\cdot}{\overset{\overset{\displaystyle H}{|}}{O}}: + \underset{\underset{\displaystyle CH_3}{|}}{\overset{\overset{\displaystyle G^{(+)}}{\|}}{C}} - O^{(-)} \rightleftharpoons HO-\underset{\underset{\displaystyle CH_3}{|}}{\overset{\overset{\displaystyle H-G^{(+)}}{|}}{C}} - O^{(-)} \xrightleftharpoons{K_4} H\underset{\cdot\cdot}{G} + CH_3COOH}$$

C

A and C = Michaelis complexes (see page 30).

B = resonance form of acetyl enzyme.

G = structure having electron transmitting properties as shown, for example, by a conjugate double bond system.

III Cholinesterase Inhibition

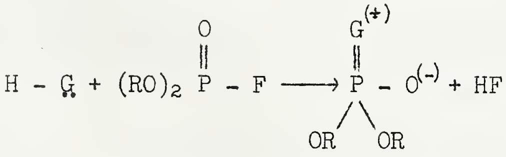
The most active types of cholinesterase inhibitors are (a) quaternary ammonium bases, (b) some of the urethanes, and (c) organophosphorus compounds. Eserine, the compound which was used in the early experiments on nerve transmission, contains a urethane group. Prostigmine, a powerful inhibitor of cholinesterase, contains a urethane group and also **is** a quaternary ammonium compound. Both these compounds are reversible inhibitors, i.e. they can be removed from the enzyme (e.g. by dialysis) which then regains its ability to hydrolyze acetylcholine (123). They are also competitive inhibitors; therefore the sequence in which substrate and inhibitor come in contact with the enzyme affects the rate but not the degree of reversible inhibition once equilibrium is established (22).

Literature concerning toxic organophosphorus compounds started to appear in 1932 (109). As early as 1937 Schrader gave the general formula for derivatives of phosphorus acids with insecticidal properties. Many of

these compounds are powerful inhibitors of cholinesterase; because of their high toxicity they are potential chemical warfare agents.

Unlike eserine and prostigmine, the organophosphorus compounds produce an inhibition which is usually irreversible (29, 97). They are, however, competitive inhibitors. For example, it was shown by Augustinsson and Nachmansohn (22) that, if acetylcholine is added before the inhibitor, a much higher concentration of antiesterase is required to produce a given degree of inhibition in a given time.

The mechanism of the inhibition reaction with organophosphorus compounds has been studied by Wilson (167, 168). Wilson and Bergman (171) postulated that the inhibitor enters the hydrolytic mechanism and phosphorylates at the esteratic site in a similar manner to the process of acetylation. They suggested the following mechanism:



(A)

Where HG̤ = esteratic site (see page 5)

A = phosphorylated esteratic site.

Although the reaction of the phosphorylated enzyme with water (reactivation) is slow, it has been demonstrated in a number of cases (11, 167). Recently, interest has been centered on compounds which accelerate the reactivation process (42, 43, 94, 165, 168, 170, 173, 174, 175). These compounds are nucleophilic agents which attach at the anionic site and

then act on the phosphorylated esteratic site.

Cohen, Oosterbaan, Warringa and Jansz (38) produced a peptide from split diisopropyl phosphorylated enzyme which contained phosphoserine. Jandorf and co-workers (95) now suggest that inhibition by organophosphorus compounds is a two-stage reaction and that both stages are irreversible The reaction postulated is a transphosphorylation from histidine to serine.

IV The Cause of Death in Anticholinesterase Poisoning

1. Acute Toxicity

The response of mammals to acutely toxic doses of anticholinesterases is concerned mainly with respiration. Barnes (26) stated that there are three major reactions, any one of which could produce death. These reactions are: (a) bronchospasm and constriction of airways, (b) interference with the conduction of impulses from motor nerves to muscles responsible for respiration and (c) interference with the initiation of impulses starting in the brain and travelling down the motor nerve supplying the muscles of respiration. The result of any or all of these reactions is asphyxia.

deCandole, Douglas, Lovatt Evans, Holmes, Spencer, Torrance and Wilson (51), in 1953, claimed that the dominant factor producing respiratory collapse was the central failure (see (c) above) due to the action of the anticholinesterase.

In 1946, Koelle and Gilman (104) showed that diisopropyl fluorophosphonate (DFP) possesses no outstanding toxic action which cannot be directly attributed to its primary effect on cholinesterase and, in 1947, Nachmansohn and Feld (143) reached a similar conclusion. With the same

compound, Freedman, Willis and Himwich (71) were able to relate the severity of toxic signs to the level of brain cholinesterase. Jones, Meyer and Karel (98) confirmed the correlation between brain cholinesterase and toxic symptoms for other organophosphorus compounds. The relationship between erythrocyte cholinesterase and toxic symptoms was less pronounced and it was non-existent in the case of plasma cholinesterase (cf also 125). It had been noted earlier (105) that the determination of serum and red cell inhibition does not provide an accurate estimate of the activity of enzyme in tissue excepting, possibly, immediately after an injection. It is now generally accepted that the respiratory symptoms are a direct result of cholinesterase inhibition (54, 89, 98, 106).

2. Chronic Toxicity

Koelle and Gilman (105) give details of long term experiments on rats, dogs and monkeys. The administration of DFP for periods up to six months produced muscle weakness, paralysis of the hind legs, urinary incontinence and spasm of the cardiac sphincter.

A delayed effect sometimes seen after an animal has recovered from an acute poisoning by certain compounds, e.g. DFP, is demyelination of the nerves in some tracts of the spinal cord. This effect leads to paralysis (49).

V Factors Modifying the Toxicity of Anticholinesterase Compounds

A series of compounds which are known to be lethal because they inhibit one particular life process may nevertheless show marked differences in toxicity. The question arises, "Why do these differences exist?". The answer to this question is particularly intriguing when one considers the

irreversible inhibitors of cholinesterase. Provided the inhibitory reaction is given enough time and no competing substance is present, equal molar quantities of such inhibitors should give the same amount of inhibition. This, of course, is a theoretical ideal not encountered in practice.

As has been stated above, there is more than one cholinesterase which competes for the inhibitor. An animal has two varieties, a specific cholinesterase which is essential to life and a non-specific which is not essential. A compound which reacts much faster with the non-specific esterase than the specific should be preferentially attached to the non-specific esterase. Such compounds exist e.g. tri-ortho-cresyl phosphate (TOCP), DFP, and N, N'-diisopropyl phosphorodiamidofluoridate (Mipafox). These substances are non-toxic in doses which inhibit only the non-specific esterase. A compound which reacts specifically with the essential esterase has not yet been found. Should such a compound be synthesized, it should be very toxic providing it reacts rapidly with the essential esterase and is resistant to destruction by other mechanisms. However, the most important factor determining the toxicity of a cholinesterase inhibitor appears to be the absolute rate of inhibition of the essential cholinesterase (see page 101). A selective inhibitor of essential cholinesterase might not be highly toxic if the rate of the inhibitory reaction were extremely slow since a considerable portion of the inhibitor could be destroyed or excreted from the body before essential cholinesterase was inhibited. DFP preferentially attacks the non-specific esterase (3, 83, 84, 103). It is, however, fairly toxic. This characteristic is probably accounted for by its rapid reaction with essential cholinesterase. Jandorf and his associates (95) give the following rate constants for DFP and two other compounds, tetraethyl pyrophosphate (TEPP)

and isopropyl methyl phosphorofluoridate (sarin):

DFP	9.5×10^4 litre mole^{-1}min^{-1}
TEPP	2.1×10^6 litre mole^{-1}min^{-1}
Sarin	6.3×10^7 litre mole^{-1}min^{-1}

The mouse toxicities for the same compounds are -

DFP	3.2 mg./kg.	(160)
TEPP	0.85 mg./kg.	(54)
Sarin	0.45 mg./kg.	(5)

The relationship between rate constant and lethal dose is apparent. Dubois and Coon in 1952 (54) also made a comparison of L.D.$_{50}$* and molar concentration for 50% in vitro inhibition of brain cholinesterase for various inhibitors. The time of incubation was not given but can be assumed to be constant for all the compounds tested and thus is a reflection of the rate constants. Their data for alkyl pyrophosphates are presented below:

Compound	L.D.$_{50}$ (mg./kg.)	Molar Concentration for 50% Inhibition
Tetraethyl pyrophosphate	0.85	4.0×10^{-9}
Dimethyl diethyl pyrophosphate	1.1	8.0×10^{-9}
Tetramethyl pyrophosphate	1.7	1.8×10^{-8}
Dimethyl diisopropyl pyrophosphate	2.5	2.0×10^{-7}
Tetraisopropyl pyrophosphate	16.0	1.4×10^{-6}

It can be seen that a relationship exists here but this relationship did not hold for alkyl thiophosphates or phosphoramides. These compounds however are in some cases metabolized to more reactive substances

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* L.D.$_{50}$ = Dose which kills 50% of the injected animals.

in the body. This of course would reduce the L.D.$_{50}$ in comparison to the molar concentration for 50% inhibition and thus, such compounds are not suitable for comparison purposes. In 1950, Dubois, Doul and Coon (55) showed that octamethyl pyrophosphoramide (OMPA) which is a weak *in vitro* inhibitor, is converted into a strong inhibitor *in vivo*. Gardiner and Kilby (75) confirmed this result. Since 1950 there have been numerous reports showing the inconsistency between *in vitro* cholinesterase inhibition and *in vivo* activity (25, 37, 52, 55, 82, 85, 89, 96, 107, 147).

The changes in inhibitory power brought about *in vivo* have been studied in four ways (36): (a) correlation of *in vitro* anticholinesterase activity with *in vivo* toxicity, (b) a comparison between the degree of tissue cholinesterase inhibition occurring *in vivo* and that resulting from the addition of the same amount of organophosphorus compound to the tissue *in vitro*, (c) a comparison of antiesterase activity before and after the incubation of a compound with a whole organ, tissue slices and homogenate, and (d) study of metabolic products recovered by partitioning between organic solvents and water or chromatographic separation. An excellent review of this subject has been published by Casida (36). In some cases, *in vivo* oxidation reduces cholinesterase activity. March and his associates (122) have shown this to be the case with some phosphates.

An important means of detoxication in the body is hydrolysis. Mazur (124) demonstrated the existence of an enzyme which catalyzed the hydrolysis of DFP. Since then, the enzymic hydrolysis of several organophosphorus compounds has been examined (17, 69, 136). This topic will be discussed in detail below in connection with the present series of experiments.

Other factors involved in toxicity are the solubility of the inhibitor and its mode of entry into the organism. For example, a relatively insoluble compound in a subcutaneous depot may be absorbed so slowly that the body can take care of it as released either by elimination, unchanged, or by some detoxicative mechanism. Similarly a compound injected intraperitoneally or administered orally may be detoxified in the liver whereas a compound injected intravenously may reach the sites of essential cholinesterase before losing much of its toxicity.

THE PROBLEM FOR INVESTIGATION

Some of the irreversible cholinesterase inhibitors are extremely toxic, others are only mildly toxic. If the toxicity is due to the anti-cholinesterase action, then some difference in metabolism must account for the differences in toxicity.

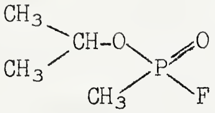
The present study was undertaken to gain some knowledge of the metabolism of two highly toxic compounds (sarin and tabun) and one mildly toxic compound (isosystox) and to evaluate the effect of metabolic processes on their toxicity.

MATERIALS AND GENERAL METHODS

I The Preparation of Sarin, Tabun and Isosystox

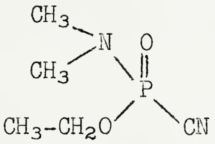
The formulae of the three compounds used in the present experiments are:

sarin



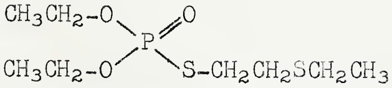
isopropyl methylphosphonofluoridate

tabun



ethyl N, N-dimethylphosphoramidocyanidate

isosystox



O, O-diethyl S-ethyl-2-mercaptoethyl phosphorothiolate

The sarin used was redistilled*. The boiling point (B.P.) was 46°C at 9 mm. and the density at 25°C (D^{25}_{4}) was 1.089.

The tabun was also redistilled. B.P. 97°C at 9 mm., D^{25}_{4} 1.071.

The isosystox was prepared in two ways. The first method is a modification of the method of Fukuto and Metcalf (72).

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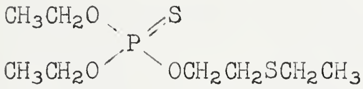
* Sarin and tabun was supplied by the Chemistry Section, Defence Research Board, Suffield Experimental Station, Ralston.

Phosphorus thiochloride was prepared according to the method of Knotz (100). For this synthesis 65 grams of phosphorus trichloride, 18 grams of sulphur and 1.8 grams of anhydrous aluminum chloride were heated in a water bath to the boiling point of the phosphorus trichloride for ten minutes. The cooled reaction mixture was placed in a separatory funnel with a large quantity of water and the phosphorus thiochloride deposited at the bottom was separated and dried with calcium chloride and distilled; B.P. 56°C at 80 mm. Yield, 56 grams, 68% of theoroetical.

O,O-diethyl thionophosphorochloridate was prepared according to the procedure described by Schrader (154). Four grams of sodium were dissolved in 250 ml. of absolute ethanol. This solution was added slowly to 56 grams of phosphorus thiochloride while the mixture was stirred with a magnetic stirrer and cooled in an ice salt bath. The mixture was then poured into 80 ml. of ice water and extracted twice with methylene chloride. The methylene chloride solution was then dried over silica gel and the solvent removed. The O,O-diethyl thionophosphorochloridate was then distilled; B.P. 125°C. Yield 32 grams, 58% of theoretical.

S-ethyl-2-mercapto ethanol was prepared according to the method of Steinkopf, Herold and Stohr (157). To a solution of 7.4 grams sodium in 125 ml. of absolute ethanol was added 20 grams of ethanethiol followed by 26 grams of chloroethane. The reaction mixture was kept moderately cool during the exothermic reaction then left overnight. The precipitated salt was removed by filtration and washed with ethanol. The ethanol was evaporated from the filtrate and the slight precipitate was removed. The S-ethyl-2-mercaptoethanol was then distilled; B.P. 184°C. Yield 20 grams, 64% of theoretical.

O,O-diethyl S-ethyl-2-mercaptoethyl phosphorothiolate was prepared by heating 32 grams of O,O-diethyl thionophosphorochloridate at 125°C with 18 grams of S-ethyl-2-mercaptoethanol, 34 grams of anhydrous potassium carbonate, 0.07 grams of copper powder and 5 ml. of benzene for four hours. The product was then distilled. By heating to 125°C any of the thiono compound



formed was isomerized completely to the thiol isomer in the reaction vessel. B.P. 93-97°C at 0.15 mm. D^{25}_{4} 1.128. Yield 4.8 grams, 11% of theoretical.

The second method of preparing isosystox was by the isomerization of commercial Systox*. A 500 gram quantity of commercial Systox was heated at 130°C for four hours. The products were subjected to distillation and the fraction boiling at 90-97°C at 0.15 mm. was collected. This liquid was redistilled; B.P. 93°C at 0.15 mm.; D^{24}_{4} 1.130. Yield about 30 grams.

The samples of isosystox prepared by both methods were tested for purity by chromatography as described by March, Metcalf and Fukuto (121). Whatman No. 1** paper was impregnated with a 5% solution of Dow Corning Corporation silicone 550 in petroleum ether. The petroleum ether was then allowed to evaporate. The upper phase of a mixture by volume

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* Obtained from the Chemagro Corp. New York.

** Other types of paper work satisfactorily e.g. Whatman 3 MM or S and S 598.

of ten parts chloroform, ten parts absolute ethanol and six parts water was used as the solvent.

As March, Metcalf and Fukuto determined the location of chromatographic fractions by radioautography of tagged material, it was necessary to devise an alternative method for locating non-radioactive material. It was decided to use a method depending on cholinesterase inhibition.

One such technique involved the use of indicators. The chromatogram was sprayed with a cholinesterase solution and subsequently with a solution of acetylcholine containing an indicator and a suitable buffer. The anticholinesterase spot remains at a more basic pH than the surrounding areas where the cholinesterase acts on the acetylcholine and thus reduces the pH. The indicator on the spots was accordingly differently coloured. As the colour differences developed by these methods were found to be extremely transitory (usually fading on drying), the method was abandoned in favour of one involving a cholinesterase substrate which would produce a colour on hydrolysis.

The method finally chosen was as follows:

The chromatogram was sprayed with a solution of purified bovine erythrocyte cholinesterase* (approximately 1,000 enzyme units per 50 ml.), or horse serum (diluted 1 to 10) and hung for five minutes in a stoppered litre volumetric cylinder which contained a layer of water in the bottom to maintain a high humidity. The strip was then taken out and sprayed with 0.01 M phosphate buffer, pH 8.4. This was immediately followed by a spray of indoxyl acetate solution which was prepared by dissolving 0.25

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* Obtained from Nutritional Biochemical Co.

grams of indoxyl acetate in 50 ml. of absolute alcohol, then making the solution up to 75 ml. with water. (The solution was warmed if precipitation occurred.) The sprayed strips were hung again in the moist cylinder. The colour developed in about ten minutes and was found to be reasonably stable if stored in the dark, preferably at refrigerator temperatures.

The results of the test on the material obtained from both methods of preparation were nearly identical and a typical strip is shown in Figure 1. The strip showed two spots, isosystox with an R_f* value of 0.66 and the sulfoxide plus sulfone (see formulae page 70-71) with an R_f value of 0.96. The R_f values given by Fukuto (73) for these substances are: isosystox 0.69, sulfoxide 0.95 and sulfone 0.98. The R_f value for isosystox was found to be affected by (a) differences in the composition of the solvent and the silicone solution used to treat the paper (e.g. March (121) used Skellysolve B as the silicone diluent wherease reagent grade ligroine was used in the present work), and (b) the type of paper used. Figure 2 shows a chromatogram with Whatman 3 MM paper. In this case the R_f value for isosystox is 0.40 and for sulfoxide-sulfone 0.77. The advantage of 3 MM paper is that its characteristics allow rapid flow.

On the assumption that the sulfoxide and sulfone were more soluble in water than was isosystox itself (solubility approximately 1:500) the product was partitioned against seven changes of water. At this point the chromatogram showed $<1\%$ sulfoxide-sulfone** (Figure 3). The purified material was dried over P_2O_5 in a desiccator.

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* $R_f = \frac{\text{Distance travelled by spot}}{\text{Distance travelled by solvent front.}}$

** Calculated as follows: amount on paper when spot barely visible ≈ 1 μgm. Amount applied to paper ≈ 250 μgms. Sulfoxide-sulfone spot is barely visible therefore it contains $< 1\%$ of applied material.

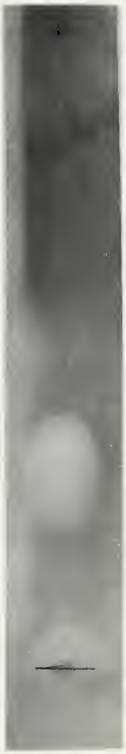


Figure 1

Chromatogram of unpurified isosystox. (Whatman #1 paper.)

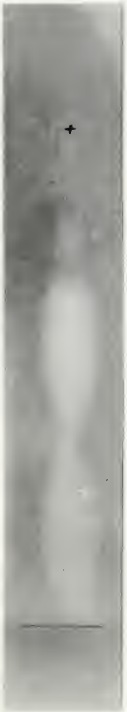


Figure 2

Chromatogram of unpurified isosystox. (Whatman #3 MM paper.)

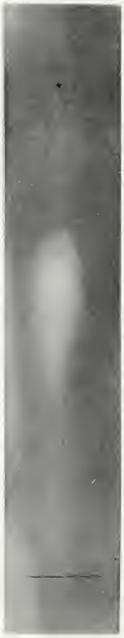


Figure 3

Chromatogram of purified isosystox. (Whatman #3 MM paper.)

+ = starting point

– = solvent front

II Estimation of Cholinesterase Activity

1. The manometric method

The Warburg method of assaying cholinesterase activity was first used by Ammon (14). This method depends on the release of carbon dioxide from a bicarbonate buffer. When acetylcholine is hydrolyzed, the following reactions take place:

$$\underset{\text{acetylcholine}}{HO(CH_3)_3NCH_2CH_2OOCCH_3} + H_2O \longrightarrow \underset{\text{choline}}{HO(CH_3)_3NCH_2CH_2OH} + \underset{\text{acetic acid}}{CH_3COOH}$$

$$CH_3COOH + NaHCO_3 \longrightarrow CH_3COONa + H_2O + CO_2$$

Details of the Warburg technique are given by Umbreit, Burris and Stauffer (164).

2. The electrometric method

The electrometric method involves the determination of pH at two or more different times. The reaction takes place in a buffer solution which must compensate for changing cholinesterase activity with changing pH. Details of the method are given by Michel (133).

Both of the above methods were used in the present work. Other techniques used for the determination of cholinesterase activity include titrimetric methods (77, 150, 156), biological methods (7) and colorimetric methods (86).

PART 1 -- THE HYDROLYSIS OF SARIN AND TABUN

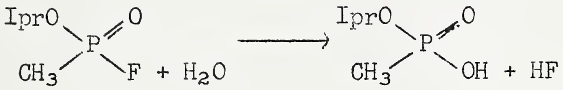
I Introduction

The first to demonstrate the enzymic hydrolysis of organophosphorus compounds was Mazur (124) who found that DFP was hydrolyzed by rabbit tissue and human blood. Later, Aldridge demonstrated the enzymic hydrolysis of another organophosphorus compound, namely, diethyl p-nitrophenyl phosphate (E600) (10). He also characterized the enzymes which hydrolyzed p-nitrophenyl acetate, propionate and butyrate into two classes (9): "A-esterases" which were not inhibited by E600 and "B-esterases" which were. He found that E600 itself was hydrolyzed by A-esterases (10). Mounter and Whittaker (141) confirmed that A-esterase was not sensitive to inhibition by organophosphorus compounds and that it hydrolyzed E600.

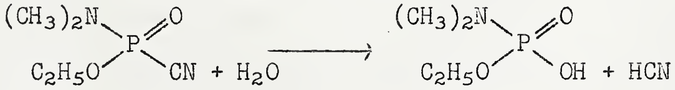
In 1953, Mounter, Floyd and Chanutin (140) purified the dialkylfluorophosphatase of hog kidney and found it to be activated by Co++ and Mn^{++}. Mounter and Chanutin, (137) obtained the formation constants for the activator-enzyme complexes. It was noted by Mounter (134) that there is more than one enzyme hydrolyzing DFP and TEPP, some of which were actually inhibited by Mn^{++} and Co^{++}. In 1954, Mounter and Dien (139), showed that hydrofluoric acid is a product of the enzymic hydrolysis of DFP.

In 1953, Adie (4) was able to show that sarin as well as DFP and E600 was hydrolyzed by an enzyme in human plasma. In one of these experiments sarin, containing P^{32}, was incubated with plasma. The reaction mixture was then subjected to electrophoresis on paper and a

radioautograph was made. Besides showing some radioactivity in the protein fractions the radioautograph showed a quantity of P^{32} not associated with any protein fraction. Subsequent investigation showed that this radioactive phosphorus was part of the isopropyl methylphosphonic acid molecule, one of the products of sarin hydrolysis (90). The equation for sarin hydrolysis is as follows:



Augustinsson and Heimburger (18, 19, 20, 21) studied an enzyme which they called "tabunase" or "phosphoryl phosphatase". This enzyme hydrolyzed tabun to give N,N-dimethylamido ethyl phosphoric acid and hydrocyanic acid as follows:



They found it to be present in the blood and other tissues of several species. In the rabbit, the adrenals, kidney and liver were most active in hydrolyzing tabun. Their work led them to suggest that the same enzyme hydrolyzed DFP. Mounter (135) stated that the dialkylfluorophosphatase of rabbit serum was an A-esterase. Similarly Goutier (80), working with radioactive DFP, found that dialkylfluorophosphatase of rabbit and guinea pig serum is identical with A-esterase. His findings were based on electrophoretic separations.

Hoskin and Trick (91) were interested in the stereospecificity of the enzyme and found it to be quite specific for the dextro-rotatory

isomer. This stereospecificity could not be demonstrated in the case of sarin hydrolysis however (6).

The following series of experiments has been designed to extend the understanding of the enzymic hydrolysis. The specificity of the enzymes involved in sarin and tabun hydrolysis will be reported. Particular emphasis will be placed on the way in which the toxicity of sarin and tabun is affected by enzymic hydrolysis in the animal.

II Experimental and Results

1. Reaction Order

The rate of an enzyme catalyzed reaction is, in general, directly proportional to the total enzyme concentration. If the substrate concentration is such that at all times the active catalytic sites on the enzyme molecule are saturated with substrate, the amount of product produced per unit time is constant. In practice it is sometimes found that the reaction rate falls off even when the enzyme is saturated, due to the accumulation of products in the solvent.

It is often necessary to use substrate concentrations which do not keep the enzyme saturated. Under these conditions the reaction may become first order, i.e. the rate of reaction is proportional to the amount of substrate present at any given time. Zero order reactions become first order when enough substrate has been converted to bring the substrate concentration appreciably below the saturation point for the enzyme. The equation for a first order reaction is

$$\text{(A)} \qquad \frac{dx}{dt} = k\ (a-x)$$

where a = the initial substrate concentration

x = the concentration of substrate converted

and k = the first order reaction constant.

Integration of this equation gives

$$\text{(B)} \qquad k\,t = 2.3 \log \frac{a}{a-x}$$

Thus for every equal interval of time, a constant fraction of the substrate is converted. If one designates the time at which 50% conversion takes place as $t_{\frac{1}{2}}$, then it follows from (B) that $t_{\frac{1}{2}} = \frac{0.69}{k}$. It is common practice to plot $\log \frac{a}{a-x}$ vs. t {or log (a-x) vs. t} as a test for a first order reaction. This should yield a straight line with slope $k/2.3$ {or $-2.3/k$ if log (a-x) vs. t is plotted}.

The non-enzymic hydrolysis of sarin and tabun was followed manometrically using the Warburg technique. The Warburg flasks contained 0.5 ml. of substrate solution in the side arm and 4.5 ml. of 0.025 M sodium bicarbonate solution in the main compartment. The rate of gas evolution at 37°C is shown in Figures 4 and 5. All substrate concentrations are expressed as the final concentration in the reaction mixture.

The total theoretical gas yield from 5 ml. of 0.01 M solution is 1278 microlitres for tabun and 2556 microlitres for sarin. This difference is explained by the fact that the HCN released from tabun is almost entirely undissociated (pk = 9.14). Accordingly, it does not contribute to the production of gas to any great extent. Thus, although 0.01 M sarin produces gas at a faster rate, the non-enzymic hydrolysis for both sarin and tabun is quite similar because sarin gives two moles of carbon

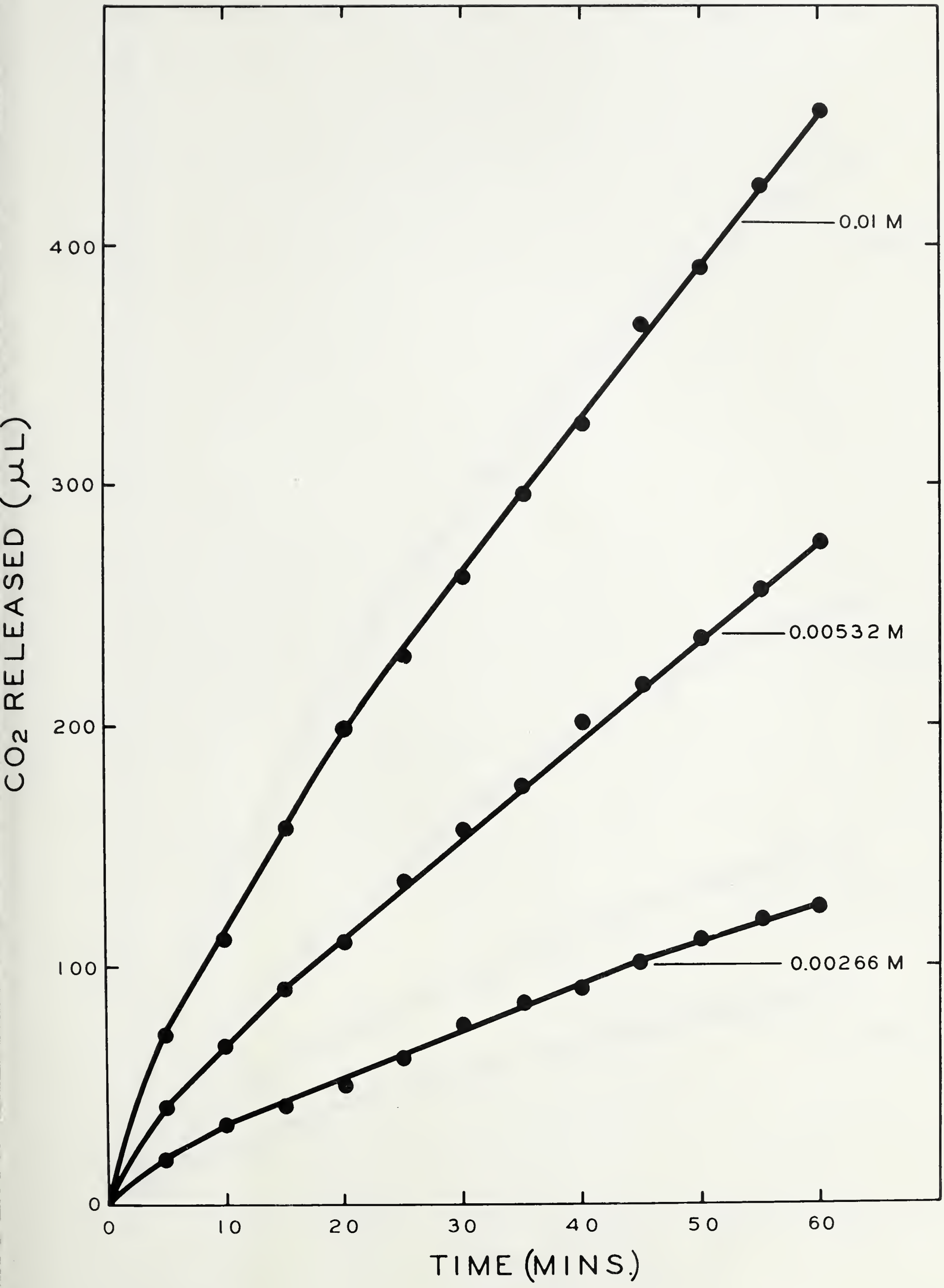


Figure 4

Non-enzymic hydrolysis of sarin
at three different substrate concentrations.

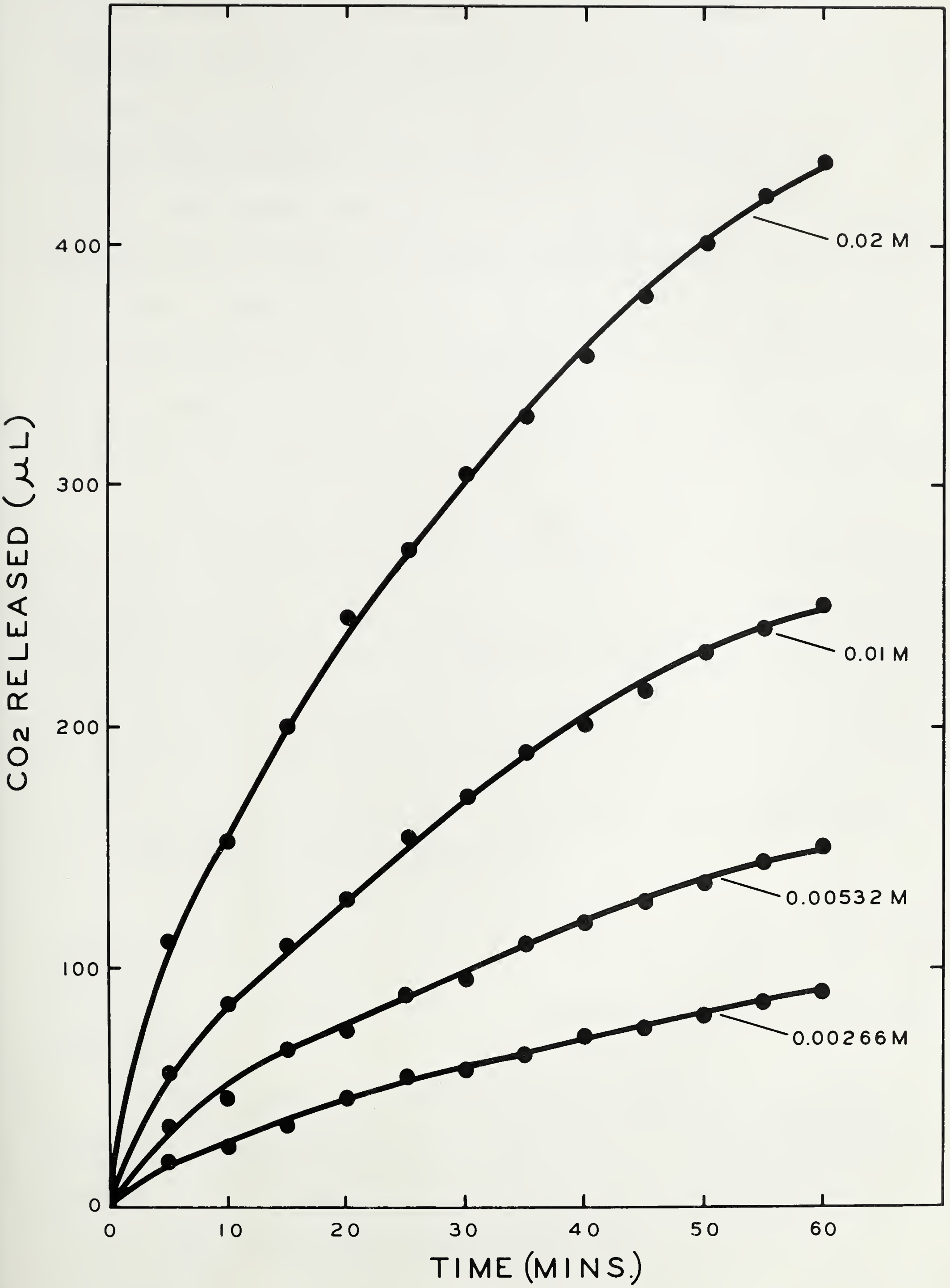


Figure 5

Non-enzymic hydrolysis of tabun at four different substrate concentrations.

dioxide for every mole hydrolyzed. In view of the rapid non-enzymic hydrolysis obtained, it was necessary to take into account the non-enzymic reaction when calculating the velocities for enzymic reaction. Accordingly in all experiments, non-enzymic controls were included.

The enzymic hydrolysis of sarin and tabun at 0.005 M and below has been shown to follow first order kinetics by Hoskin and Trick (91) and by Adie, Hoskin and Trick (6). To be sure that this applied over the range of substrate concentrations used in the following experiments it was necessary to check the reaction order at 0.04 M substrate concentration. The enzyme source chosen for this experiment was rat serum.

The Warburg flasks contained 0.5 ml. of 0.4 M sarin, 0.5 ml. of rat serum diluted 1:5 with isotonic saline solution and 4.0 ml. of bicarbonate buffer. A graph showing the gas evolution at 37°C is seen as Figure 6. It appears from this graph that, initially, the reaction approximates zero order. However, a plot of log a-x vs t (Figure 7) shows that the reaction is first order. The figure for "a" is taken to be the maximum theoretical gas yield at this concentration (10224 microlitres).

2. The Effect of Substrate Concentration

As substrate concentration is increased from low to high concentrations the reaction kinetics change from first order to zero order. The change in velocity is actually an asymptotic approach to a limiting value "V_{max}".

In 1913, Michaelis and Menten (132) formulated a mathematical analysis of enzyme action which is still, in its essentials, generally accepted. A brief outline of this formulation is given below.

The reaction considered is as follows:

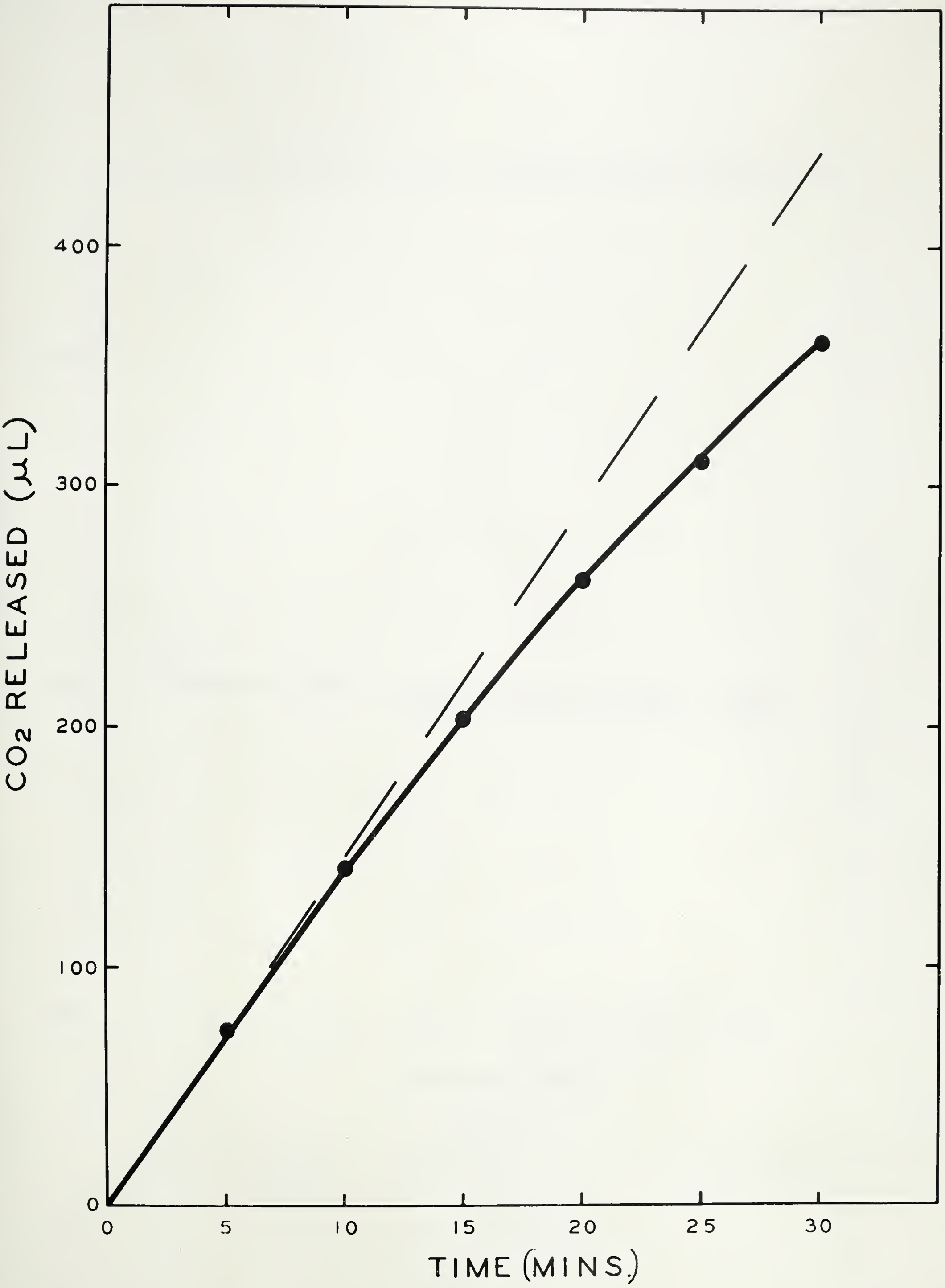


Figure 6

Enzymic hydrolysis of sarin

The broken line shows the initial rate extrapolated to 30 minutes.

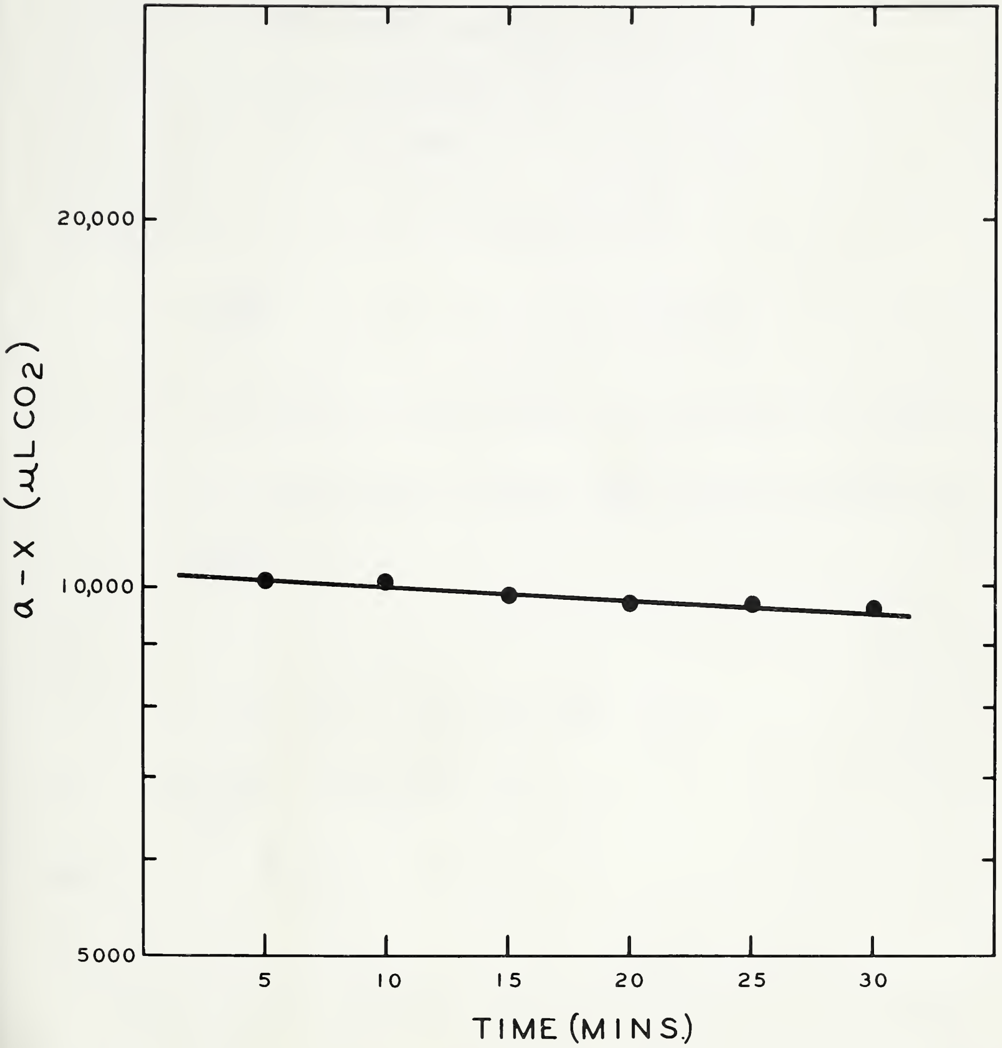


Figure 7

Enzymic hydrolysis of sarin plotted as first order reaction

a = 10224 μl.

$$E + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_3} E + P$$

where E = enzyme in the free state

ES = enzyme substrate complex

S = substrate

P = products.

Assuming there is an excess of substrate then

$$\frac{d\,[ES]}{dt} = K_1\,[E]\,[S] - (K_2 + K_3)\,[E\,S]$$

where the concentration brackets denote the concentration at time t.

If a steady state as regards $[ES]$ has been achieved then

$$\frac{d\,[ES]}{dt} = 0$$

and

$$K_1\,[S]\,[E] - (K_2 + K_3)\,[ES] = 0$$

or

$$\frac{[S]\,[E]}{[ES]} = \frac{K_2 + K_3}{K_1} = K_m$$

where K_m = Michaelis constant.

Points of interest about this enzyme constant are:

a. K_m has the dimensions of concentration, i.e. moles/litre.

b. When the substrate concentration $[S]$ equals the Michaelis constant (K_m), the velocity of the reaction (v) is one half the maximum velocity (V_{max}).

c. The equation has been derived for negligible $[P]$.

d. Once K_m is known, the per cent V_{max} can be calculated for any $[S]$.

Substituting v for K_3 $[ES]$ and assuming $[ES] << [S]$ the above equation for the Michaelis constant becomes

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

Lineweaver and Burk (111) rearranged this equation to give the following:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(\frac{1}{[S]}\right) + \frac{1}{V_{max}}$$

This is an equation of the form $y = ax + b$. A plot of $\frac{1}{v}$ vs. $\frac{1}{[S]}$ produces a straight line with a y intercept of $\frac{1}{V_{max}}$ and a slope of $\frac{K_m}{V_{max}}$.

The proof that an ES complex existed was provided in the late 1930's by Stern (158), Keilin and Mann (99) and Theorell (163) who worked with heme enzymes. An excellent review of enzyme kinetics in general has been presented by Alberty (7).

The Michaelis constants for the enzymic hydrolysis of sarin and tabun were measured by means of the Warburg technique. Six different concentrations of the two substrates were used for this purpose. The reaction flasks contained 0.5 ml. of rat serum, diluted 1 in 5, 0.5 ml. of substrate solution (in the side arm) and 4.0 ml. of 0.025 M sodium bicarbonate solution. The determinations were carried out at 37°C. The data are represented in Figures 8 and 9. Figure 10 is a graph showing the relationship between $\frac{1}{v}$ and $\frac{1}{[S]}$. The value of v was obtained by extrapolating the initial velocity to 30 minutes, i.e. the amount of CO_2 that would be evolved in 30 minutes if the initial reaction rate were maintained.

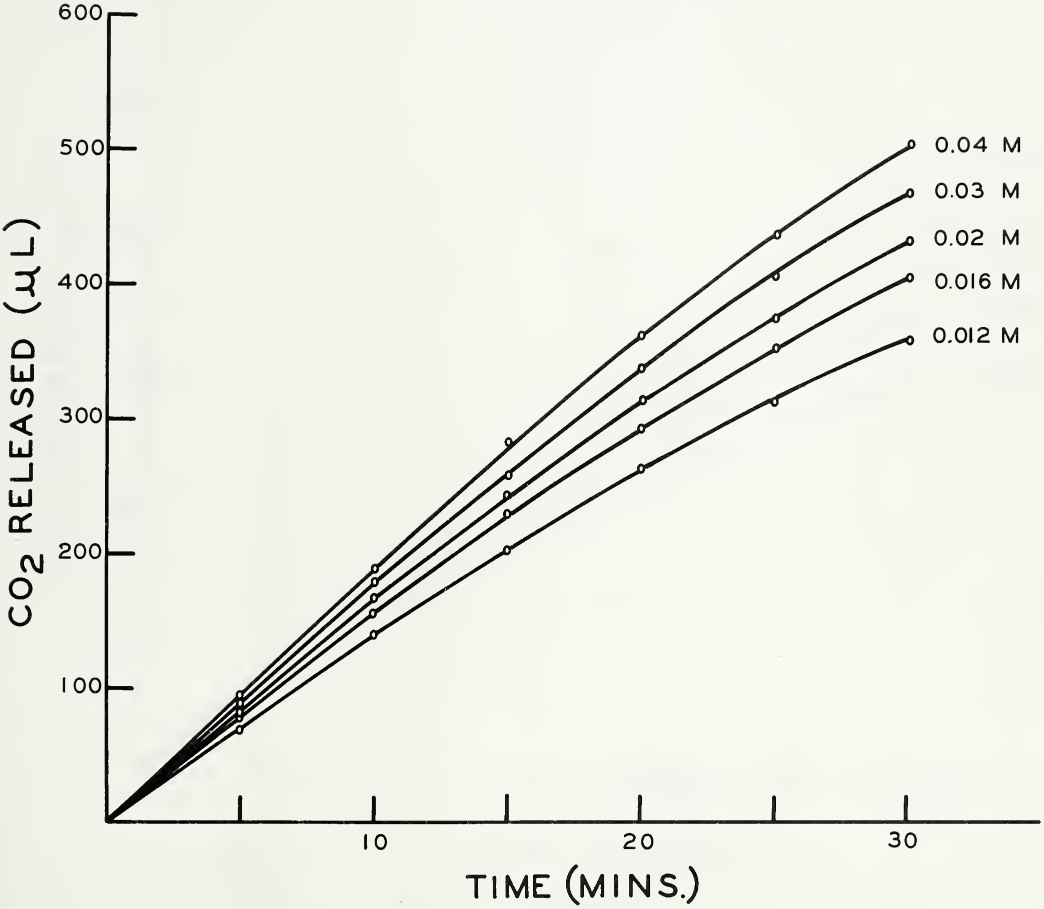


Figure 8

Effect of substrate concentration on the hydrolysis of sarin by rat serum

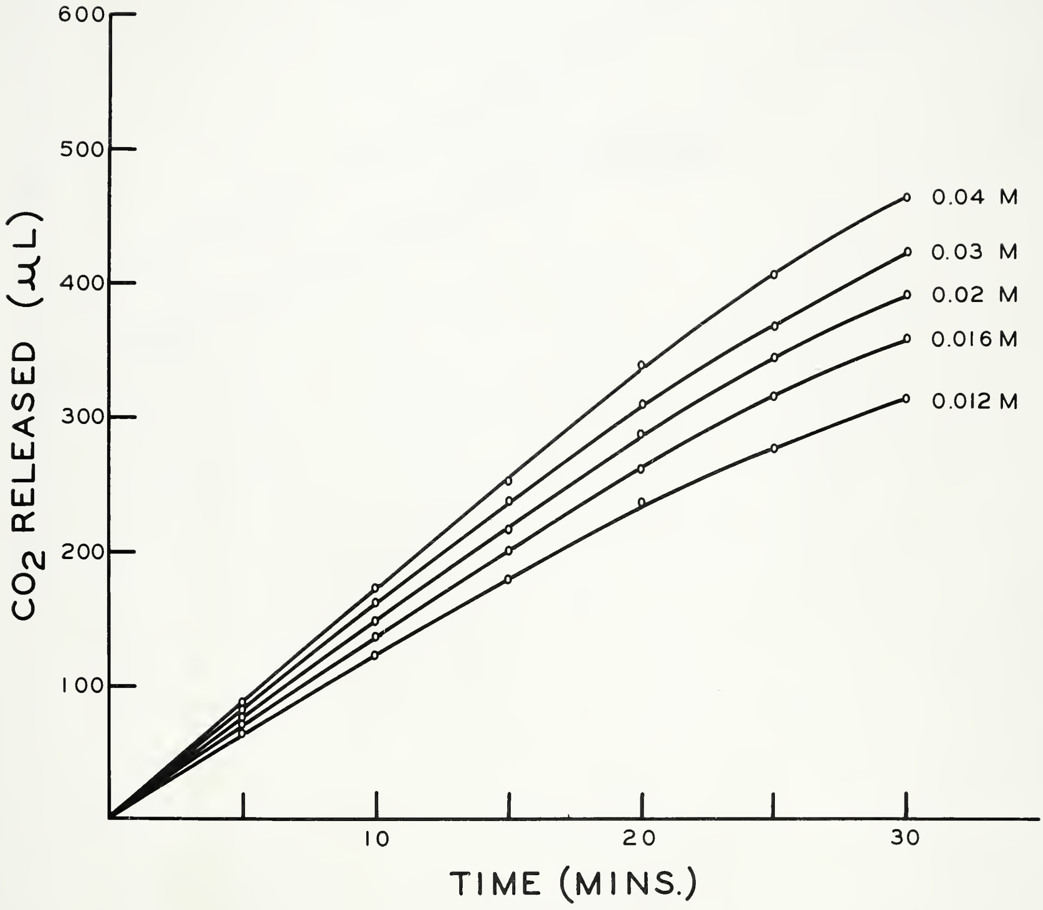


Figure 9

Effect of substrate concentration on the hydrolysis of tabun by rat serum.

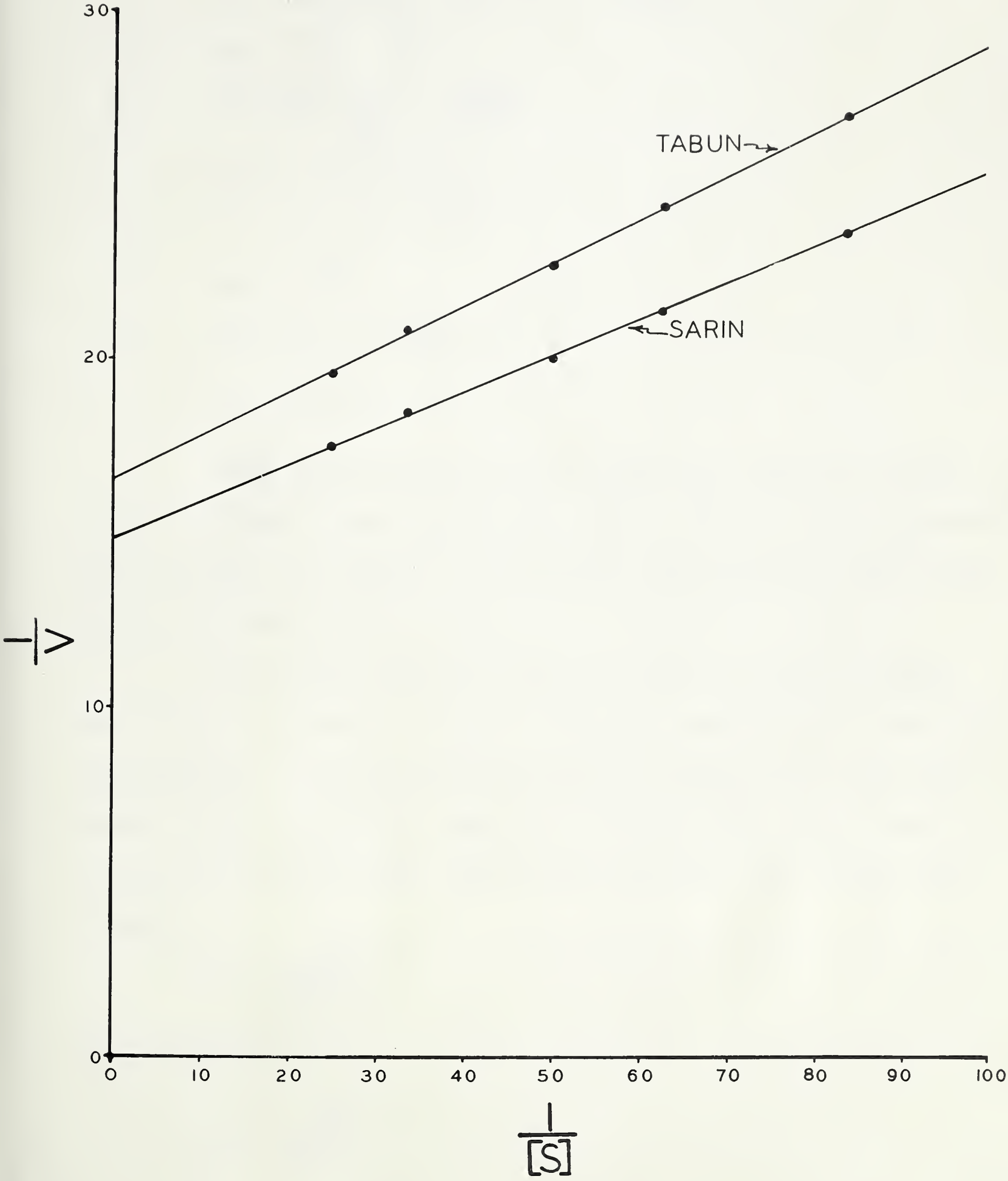


Figure 10

Plot for obtaining Michaelis-Menten constants for the hydrolysis of sarin and tabun.

From Figure 10 Michaelis constants were obtained.

The intercept $\frac{1}{V_{max}}$ for sarin = 0.00149

$V_{max} = 671$

The slope $\frac{K_m}{V_{max}} = \frac{0.00105}{100}$

$\therefore K_m = 7.0 \times 10^{-3}$ M.

For tabun $\frac{1}{V} = 0.00165$

$V_{max} = 606$

The slope $= \frac{0.00125}{100}$

$\therefore K_m = 7.6 \times 10^{-3}$ M.

These constants are of the same order of magnitude as those obtained by Augustinsson and Heimbürger (19). They used horse plasma and purified human blood enzyme and obtained K_m values of 3.45×10^{-3} M and 2.15×10^{-3} M tabun respectively.

As mentioned above, the value of K_m can be used to calculate the rate of destruction of sarin at any given concentration of sarin. The concentrations of sarin in the animal will be extremely low since only minute amounts of sarin are required to kill the animal (approximately 15 µg./kg. or 10^{-7} M sarin). Under these conditions, i.e. $[S] << K_m$, the equation

$$v = \frac{V_{max}\,[S]}{[S] + K_m}$$

can be simplified to

$$v = \frac{V_{max}\,[S]}{K_m} \quad .$$

For any given value of V_{max} (i.e. for any given amount of a particular

sample of serum), the rate of hydrolysis of sarin (v) will then be directly proportional to the concentration of sarin $[S]$. The kinetics of the hydrolysis will therefore be first order and the half life of sarin will be independent of the concentration $[S]$.

In the case of the sample of rat serum used for the above experiments, $K_m = 7 \times 10^{-3}$ M sarin and $V_{max} = 671$ µl. CO_2/30 min. with 0.5 ml. of five-fold diluted rat serum in a total volume of 5 ml. This value for V_{max} is equivalent to 6.71×10^6 µl. CO_2/30 min. or 5×10^{-3} moles of sarin per minute per litre of undiluted rat serum. Substituting the values of K_m and V_{max} in the above equation

$$v = \frac{5 \times 10^{-3}\ [S]}{7 \times 10^{-3}} = 0.71\ [S]\ \text{min.}^{-1}$$

Because $v = k[S]$ for a first order reaction this rate constant k is therefore 0.71 min.$^{-1}$. Substituting this value in the equation $t_{\frac{1}{2}} = 0.69/k$ given above (see page 24), it is seen that the half life of sarin in undiluted rat plasma is 0.97 minutes, provided the concentration of sarin is much less than 7×10^{-3} M. The sarinase therefore represents an active mechanism for the detoxication of sarin in the body; the effect of this detoxication on the toxicity of sarin will be considered later in this section.

3. The Activation Energy for the Enzymic Hydrolysis of Sarin and Tabun

Arrhenius (76) derived empirically an equation which describes the effect of temperature on the velocity of a reaction as follows:

$$k = Ce^{-A/RT}$$

"A" represents the activation energy, i.e. the energy the mole-

cules have to acquire in order to be capable of undergoing reaction. The term C is usually known as the frequency factor.

Another form of the integrated Arrhenius equation is

$$\ln K = \frac{-A}{R}\left(\frac{1}{T}\right) + \text{Constant}$$

"A" can now be evaluated by plotting log K vs $\frac{1}{T}$ for several temperatures. The slope of this graph is -0.219 A. For first order reactions, the units of "A" are calories and for second order reactions, calories per mole. The catalytic effect of an enzyme on a reaction is brought about by the formation of an intermediate complex. This complex formation reduces the activation energy required.

The rates of hydrolysis of sarin and tabun at different temperatures were investigated using bovine plasma as the enzyme source. The Warburg technique was used for this experiment. All temperatures were controlled to within ± 0.01°C.

The reaction vessels contained 0.5 ml. of bovine plasma, 4.0 ml. of 0.025 M bicarbonate buffer and 0.5 ml. of 0.1 M substrate. At the higher temperatures the time was reduced because of enzyme denaturation and high gas evolution. The results are plotted in Figures 11 and 12. From these graphs the first order reaction constants were obtained. The logarithms of these constants were plotted graphically against the reciprocal of the absolute temperature (Figure 13).

From this graph the activation energies were calculated from the equation, -0.219 E = Slope, as follows:

$$\text{For sarin:} \quad A = \frac{2.000}{0.0006 \times 0.219} = 15{,}000 \text{ cal.}$$

Figure 11

The effect of temperature on the hydrolysis of sarin.

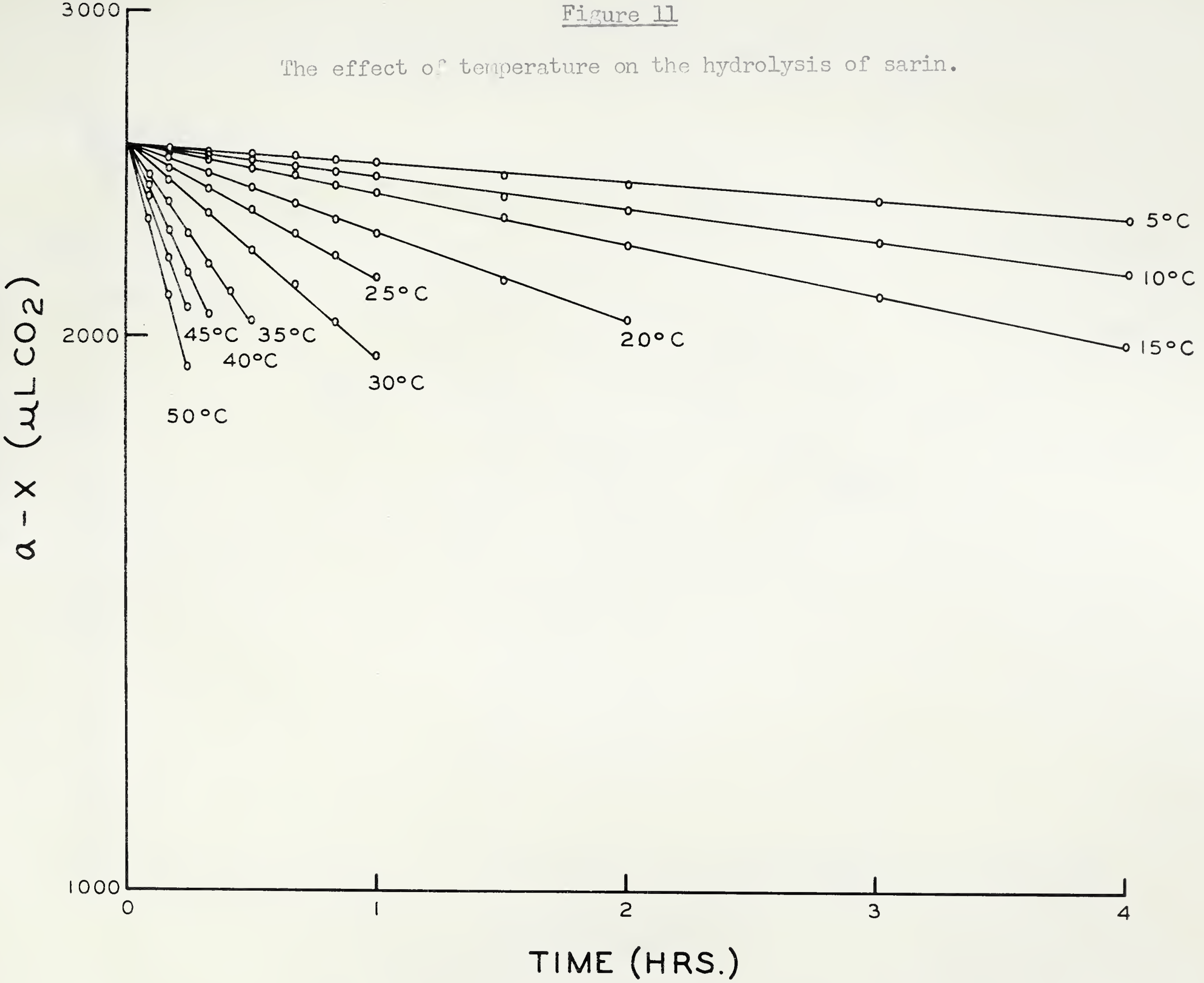
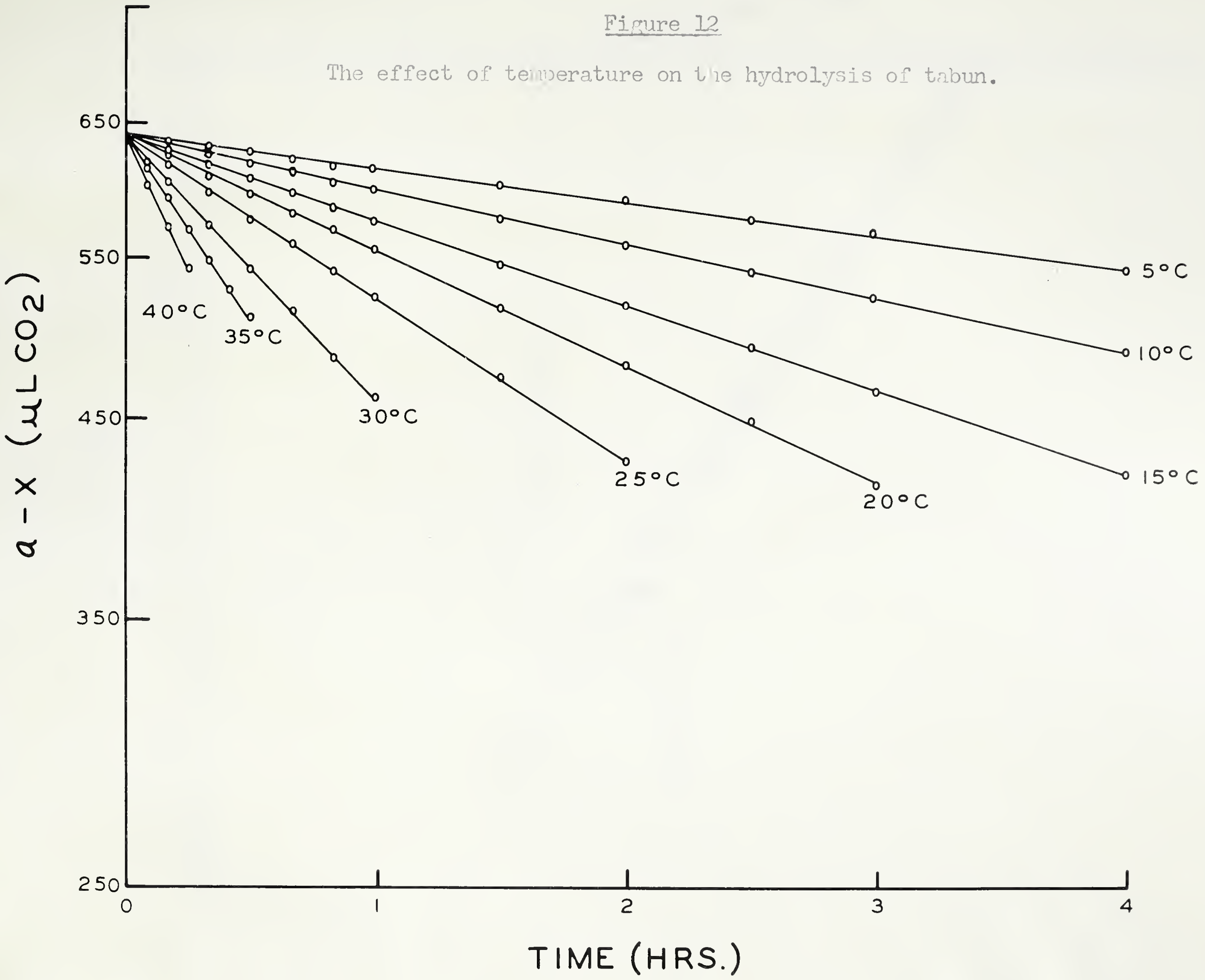


Figure 12

The effect of temperature on the hydrolysis of tabun.



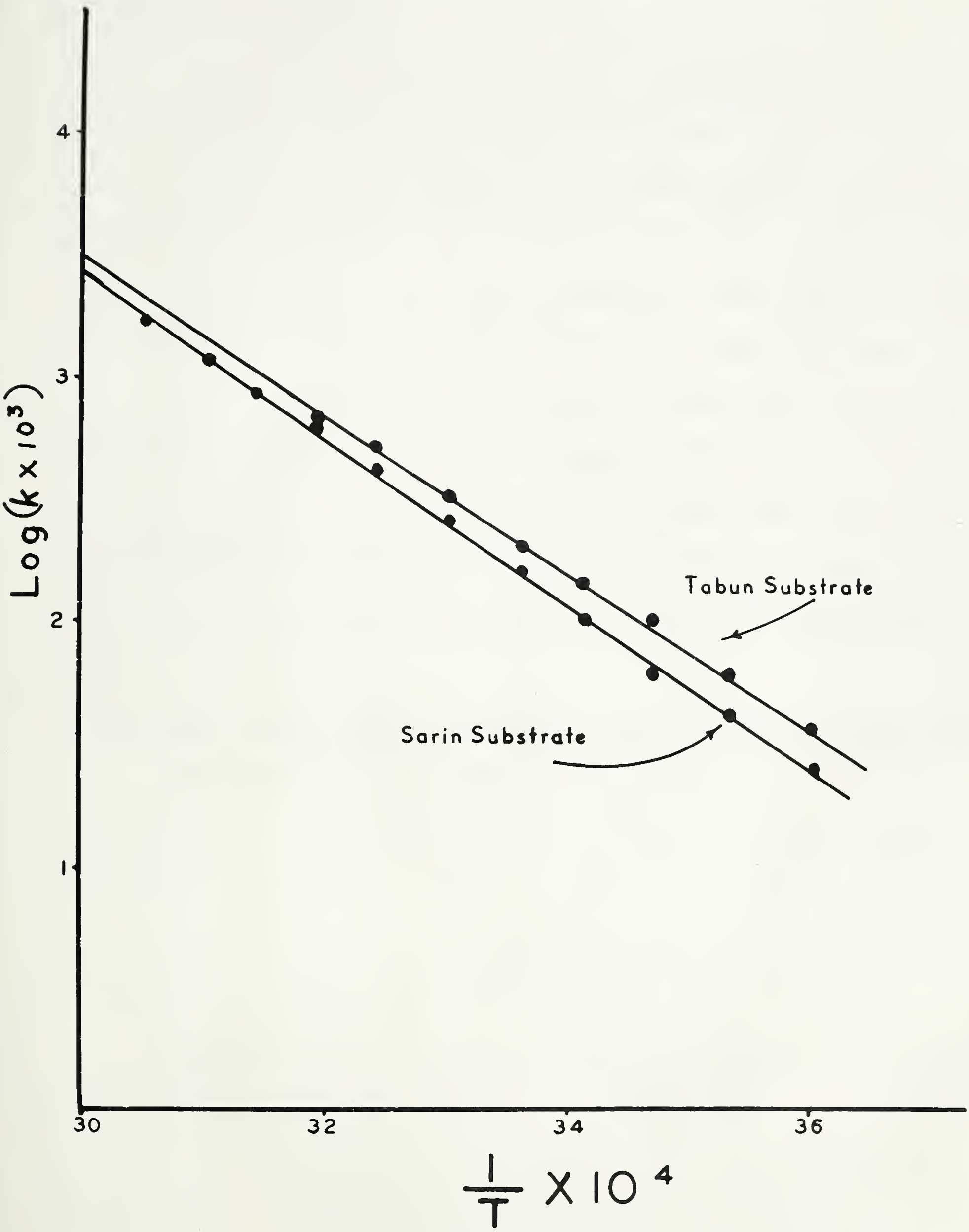


Figure 13

Effect of temperature on the rate constants for the hydrolysis of sarin and tabun.

For tabun: $A = \frac{1.9000}{0.0006} \times 0.219 = 14,600$ cal.

4. Thermal Inactivation

A marked difference in the rate of heat inactivation of sarinase and tabunase would indicate separate enzymes, but similarity in the rates of heat inactivation would suggest that a single enzyme was involved. Accordingly, bovine plasma was incubated for 14 hours at various temperatures. The plasma was then tested in duplicate experiments for hydrolytic activity with both sarin and tabun by the usual manometric method.

The hydrolysis rates, calculated as percentages of the rate obtained with controls which were kept refrigerated at 4°C during the incubation period, were plotted graphically (Figure 14). The "t" test for significance was applied to the data for sarin and tabun paired at each temperature. No significant difference was found even at the 0.1 level.

5. Effect of Hydrogen Ion Concentration on the Stability of the Enzyme

Because of the instability of the enzyme and substrates at alkaline pH and of the enzyme at acid pH, it was found that the pH optimum was difficult to determine. However, differences in enzyme denaturation after incubation at different hydrogen ion concentrations could be used to indicate that sarinase and tabunase were different enzymes.

Duplicate samples of bovine plasma were incubated for fourteen hours at 37.5°C in Michaelis "universal" buffers (131) ranging from pH 4.5 to pH 9.6 and in a borate buffer at pH 10.5. These samples were then dialyzed against isotonic saline to remove the buffer and tested for hydrolytic activity with both sarin and tabun using the Warburg technique

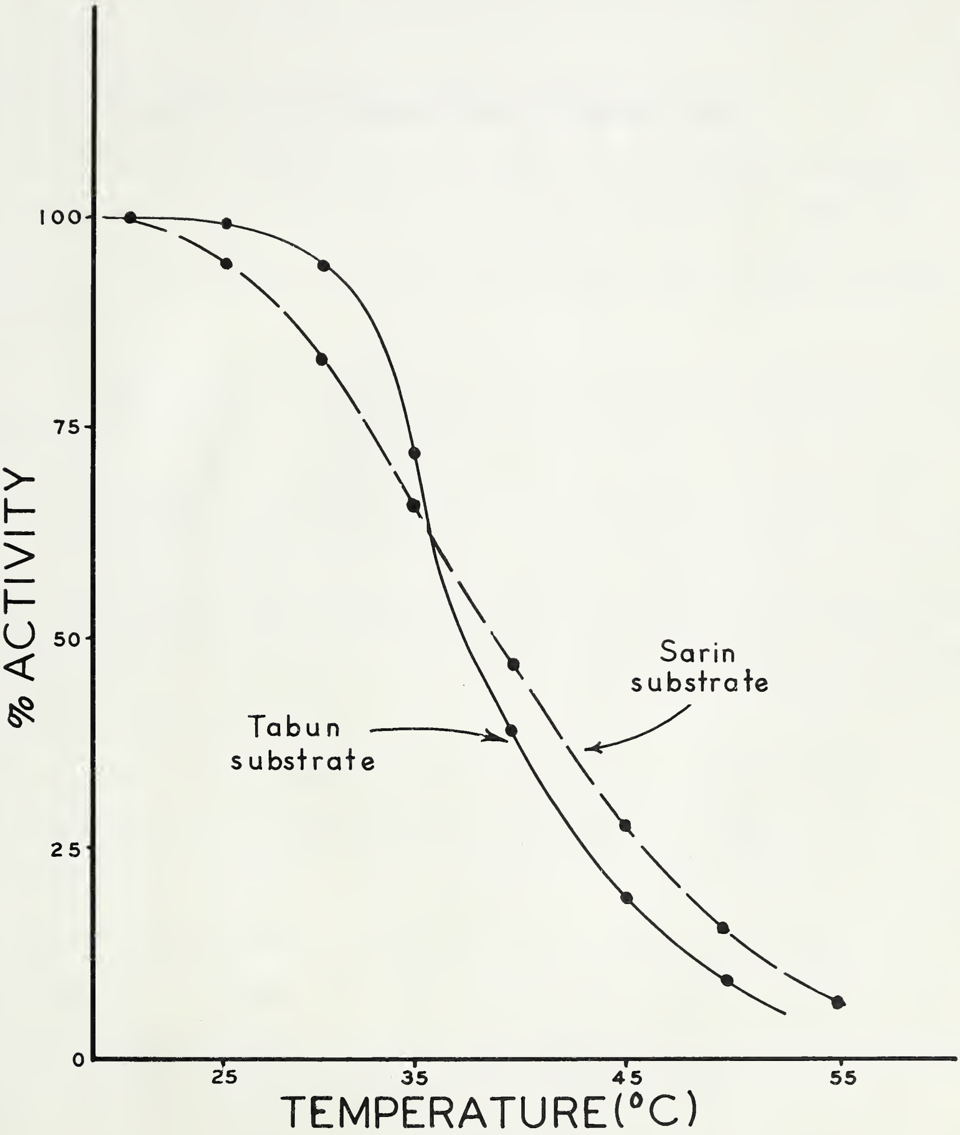


Figure 14

Thermal inactivation of sarinase and tabunase.

as described above. The resulting extrapolated initial activities, recorded as a percentage of normal plasma activity, are shown graphically in Figure 15. In this case there was very close agreement in activity towards the two substrates.

6. The Purification of the Enzyme Which Hydrolyses Sarin

The method chosen for purifying the sarin hydrolysing enzyme from bovine plasma was that used by Cohn and his associates (40) for the fractionation of human plasma. The procedure can be seen in outline in Figure 16.

Each fraction obtained was tested for sarinase activity in the Warburg apparatus in the usual manner. An aliquot was retained for nitrogen determinations. In order to remove the last traces of alcohol and ensure that the solutions tested were of equal ionic strength, all fractions, before being tested, were dialyzed for at least fourteen hours with internal stirring against four or more changes of physiological saline. The results are shown in Table I.

Fraction VI-2 was centrifuged for 14 hr. at 40,000 r.p.m. (105,400 x g.) in a preparative ultracentrifuge. The upper two-thirds and lower one-third of the solution in the centrifuge tubes were tested for sarinase activity. A small amount of precipitate which adhered to the walls of the tubes was not tested. The lower portion had a specific activity of 4625 µl.CO_2/mgm.N/30 min., which is 1250 times the activity of the original plasma. The upper portion exhibited no sarinase activity.

Figure 17 shows the electrophoretic patterns of fractions VI, VI-1 (Table I) and fraction VI-2 (lower). The solutions were concentrated by freeze-drying in order to obtain these patterns.

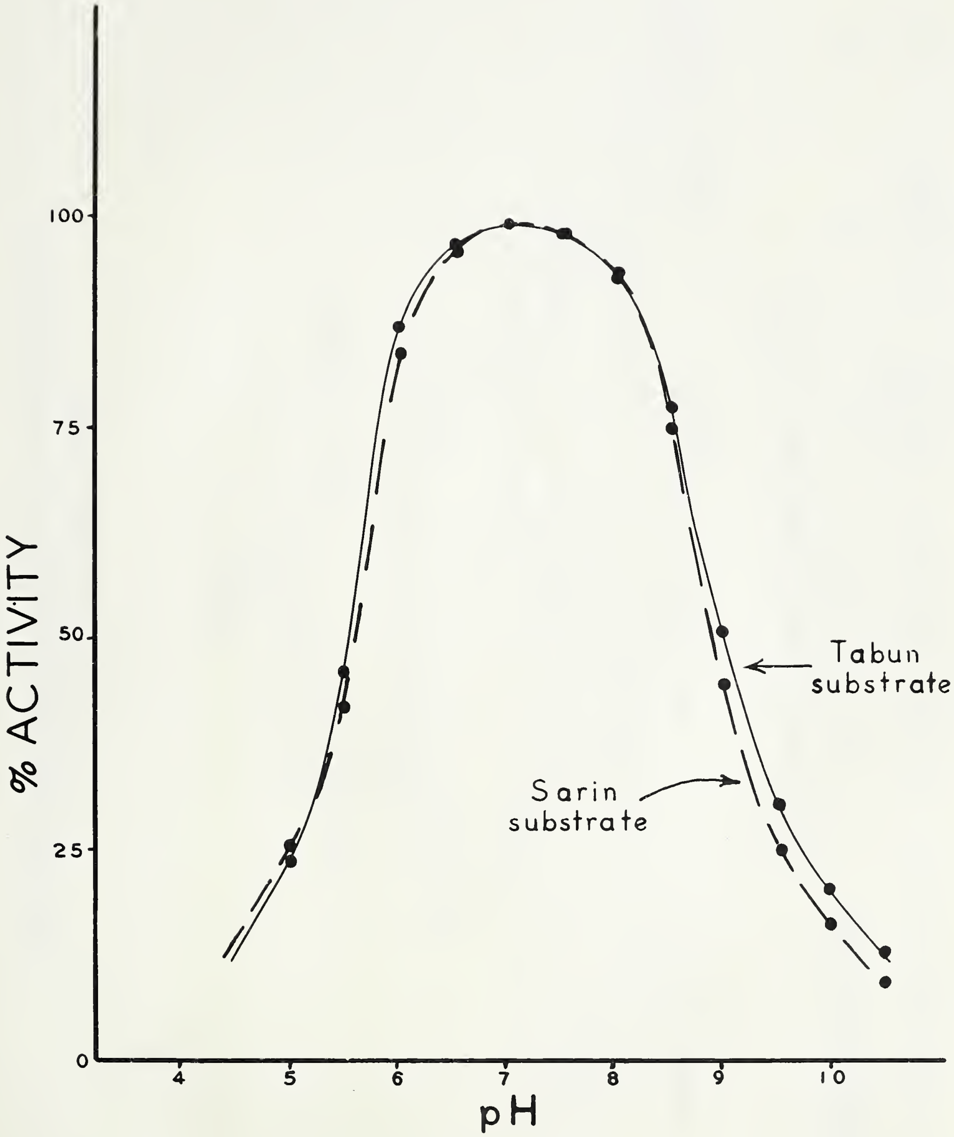
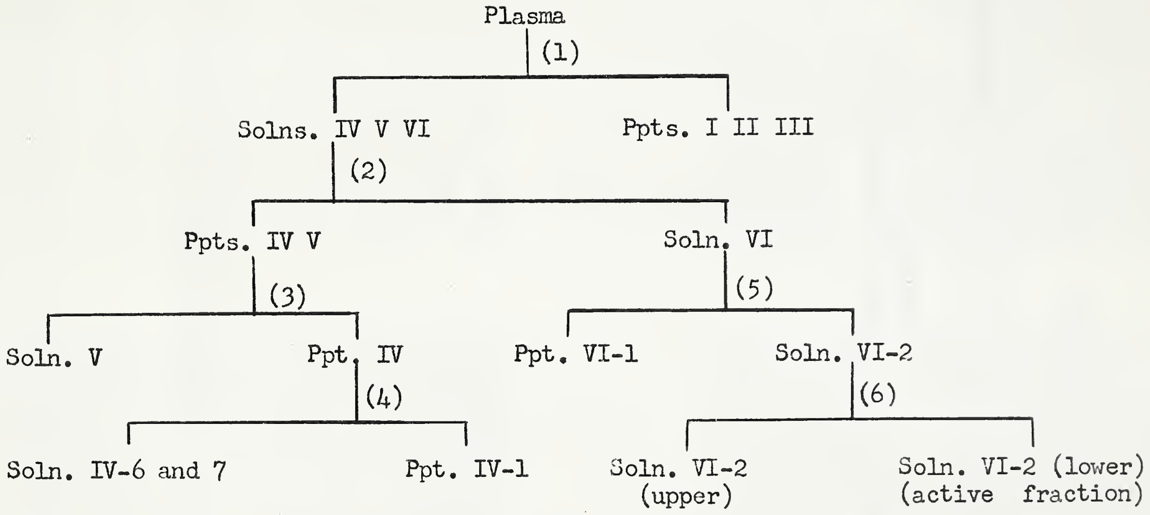


Figure 15

Inactivation of enzyme by incubation at various pH values.

Figure 16

Flow sheet showing the procedure for the purification of sarinase from bovine plasma



Conditions for Separations (Temp. = -5°C)

Separation number (refers to diagram)	1	2	3	4	5	6
Conc. of metal (moles/litre)	Na (0.04)	Zn (0.02)	Zn (trace) Ba (0.01) Na (0.02)	Zn (0.0005) Na (0.05)	Ba (0.02)	Ultra-centrifuge
pH	5.8	5.8	5.5	6.2	6.1	
Ethanol (mole fract.)	0.066	0.066	0.051	0.051	0.066	

Table I

Specific Sarinase Activity of Protein Fractions of Bovine Plasma

Fraction*	Specific Activity** (μl.CO_2/mgm.N/30 min.)	Activity of protein fraction/ Activity of plasma
Plasma	3.7	1.00
I II III	1.4	0.38
IV-1	5.3	1.43
IV-6 and 7	7.6	2.05
V	24	6.49
VI	330	89
VI-1	9.3	2.5
VI-2	893	241

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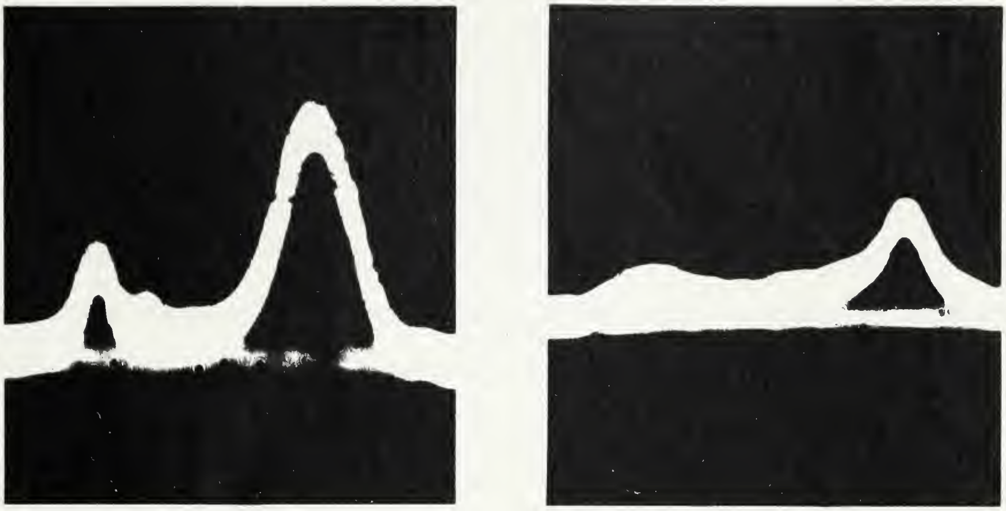
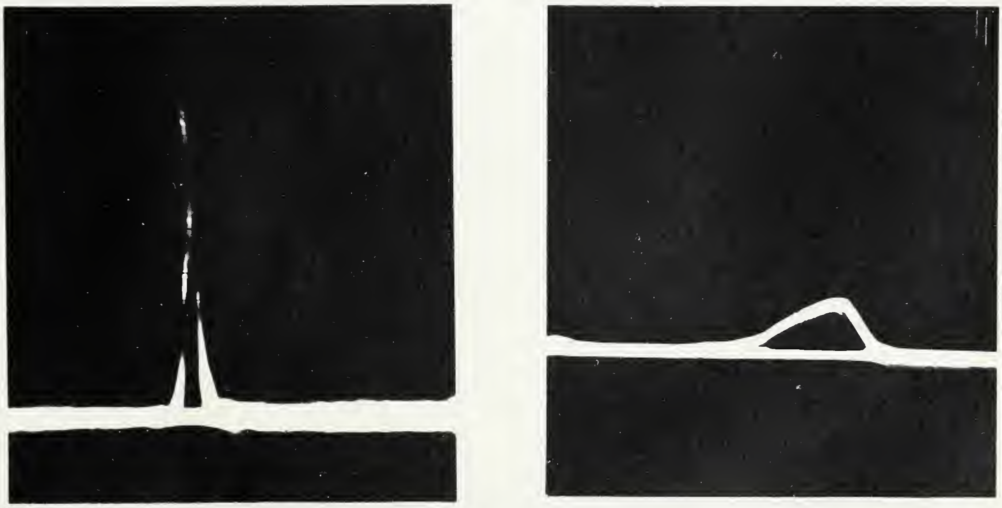
* Cohn's human plasma fraction numbering.

** Nitrogen determined by micro-Kjeldahl method (70)



Fraction VI
Ascending boundary

Fraction VI-1
Ascending boundary



Fraction VI-2 (lower)
Descending boundary

Fraction VI-2 (lower)
Ascending boundary

Figure 17

Electrophoretic patterns of fractions.

Dialysis of fraction VI-2 (lower), freeze-drying, and subsequent solution in physiological saline reduced the specific sarinase activity by 60%.

The purified preparation (VI-2 lower) was tested for activity against tabun as it was previously for sarin i.e. by the Warburg method. The activity was found to be 1150 times greater than the activity of the original plasma. This result indicated that the same enzyme was concerned in the hydrolysis of both sarin and tabun.

The sarinase activity is concentrated in Cohn's fraction VI-2. In human plasma this fraction contains α_1-lipid free glycoprotein of small molecular weight, with an isoelectric point about pH 3 (40). The specific activity of this fraction was increased many times by ultracentrifugation. This would suggest that more than one protein was present in fraction VI-2. This was borne out by the electrophoresis pattern obtained. It is possible that much of the increase in specific activity was due to the removal of the material which adhered to the walls of the ultracentrifuge tubes as well as to the sedimentation of the sarinase. The electrophoresis pattern of fraction VI-2 (lower) showed that this material was apparently homogeneous. The sharp peak produced by the descending boundary is similar to the β anomaly encountered with the descending boundary of human plasma (2). This phenomenon does not occur with whole bovine plasma (88).

7. Distribution of Hydrolyzing Enzymes in Mammalian Tissue

Various tissues (homogenized where necessary at 0°C in a Potter homogenizer) were tested manometrically at 37°C for hydrolyzing activity with both 0.01 M sarin and 0.01 M tabun as substrates. From plots of

CO_2 production against time, initial reaction rates were obtained. Table II lists the means with standard errors of the initial reaction rates (expressed as µl. CO_2 per hour) for each tissue.

There is a wide variation in the enzyme levels between individuals in a species (shown by the standard errors). There are also variations in the amount of enzyme in the different tissues of each species. The activity of the liver of all species is shown to be higher in activity than that of the plasma for sarin hydrolysis. The intestinal mucosa and kidney are very active against both sarin and tabun. On a weight basis the highest activity with both substrates is shown by rabbit liver and the second highest by rat liver.

An interesting point brought out by this table is the fact that the ratio of sarin to tabun hydrolysis was not consistent. Accordingly, ratios were taken of the sarin to tabun hydrolysis of the individual animals. The means and standard errors were calculated and are listed in Table III. It can readily be seen that the relative rates of hydrolysis are dependent on the enzyme source.

8. The Intracellular Localization of Liver and Kidney Sarinase and Tabunase

It will be shown that the amount of sarinase in the liver has an effect on the resistance of individual rabbits to intraperitoneally injected sarin. It seems possible that the large differences in the susceptibility of different species to sarin may be due to differences in the amount of sarinase present in the tissues. Moreover, if the sarinase were more effective in one cellular fraction than another, the distribution of sarinase within cells might have some bearing on sarin tolerance.

Table II

Hydrolysis Rates of Sarin and Tabun Incubated with Various Tissues

Enzyme Source		Number of Animals	Sarin Hydrolysis (μl.CO$_2$/hr. ± Std.error) Tissue sample = 0.1 gm.	Tabun Hydrolysis (μl.CO$_2$/hr. ± Std. error) Tissue sample = 0.1 gm.
Monkey	liver	5	777 ± 191	338 ± 74
	plasma	5	44 ± 10	37 ± 7
Cat	liver	2	1413 ± 122	314 ± 30
	plasma	2	147 ± 13	70 ± 6
Dog	liver	2	1103 ± 300	234 ± 72
	plasma	2	220 ± 51	129 ± 24
Guinea pig	liver	3	1134 ± 117	406 ± 64
	plasma	3	300 ± 59	272 ± 50
Rat	liver	9	4840 ± 436	1008 ± 76
	plasma	9	515 ± 43	414 ± 32
	kidney	9	1575 ± 131	450 ± 39
Rabbit	liver	6	7640 ± 1136	1776 ± 216
	plasma	6	900 ± 139	900 ± 127
	intestinal mucosa	2	2260 ± 319	1130 ± 154
	brain	2	63 ± 8	56 ± 7
	kidney	2	3311 ± 530	2365 ± 408
	adrenals	2	442 ± 63	340 ± 48
	spleen	2	1042 ± 98	680 ± 61

Table III

Ratios of Initial Hydrolysis Rates with Sarin and Tabun Substrates

(0.01 M)

Enzyme source		Number of animals tested	Activity Ratio sarin/tabun (mean ± standard error)
Monkey	liver	5	2.3 ± 0.2
	plasma	5	1.2 ± 0.2
Cat	liver	2	4.5 ± 0.5
	plasma	2	2.1 ± 0.3
Dog	liver	2	4.7 ± 0.4
	plasma	2	1.7 ± 0.2
Guinea pig	liver	3	2.5 ± 0.5
	plasma	3	1.1 ± 0.2
Rat	liver	9	4.8 ± 0.2
	plasma	9	1.2 ± 0.1
	kidney	9	3.5 ± 0.1
Rabbit	liver	6	4.3 ± 0.2
	plasma	6	1.0 ± 0.2
	intestinal mucosa	2	2.0 ± 0.4
	brain	2	0.9 ± 0.1
	kidney	2	1.4 ± 0.3
	adrenal	2	1.3 ± 0.3
	spleen	2	1.5 ± 0.3

This series of experiments was designed to reveal any variations in the intracellular distribution of sarinase, both between the same organs from different species and between different organs from the same species. It was not designed to reveal differences in total activity between species, as total activity was affected by time of storage of the organs after their removal from the animal. Three species susceptible to sarin on the basis of $L.D._{50}$ (rabbit, guinea pig and monkey) and two less susceptible species (rat and mouse) were chosen for comparison. A similar degree of activity, towards both sarin and tabun, by cell fractions would indicate that one enzyme is responsible for the hydrolysis of both substrates. Accordingly, experiments testing this hypothesis were carried out also.

(a) Cell fractionation

Animals were stunned by a blow on the back of the head (monkeys were first anaesthetized) and killed by decapitation to ensure maximum removal of blood from the organs. The two kidneys and the liver were removed at once from each animal. The tissues were frozen and stored if they were to be homogenized at a later date. Otherwise the tissues were rinsed in ice-cold isotonic (0.25 M) sucrose solution, blotted with filter paper and cut into smaller pieces with scissors. A weighed amount of the tissue was homogenized with isotonic sucrose in a Potter glass homogenizer, which was cooled in crushed ice, and the homogenate was made up to a volume of 10 ml. with sucrose. One ml. of the homogenate was put aside for estimation of total enzyme activity of the unfractionated tissue. The remaining 9 ml. were centrifuged at 2,000 r.p.m. (600 x g.) at about 2°C for 10 minutes. The supernatant liquid was decanted, while

the first precipitate (nuclear fraction) was resuspended and washed twice with cold sucrose, and the washings, after centrifugation were added to the first supernatant. The combined supernatants were then centrifuged at 9,500 x g. and at 2°C for 10 minutes. The second precipitate (mitochondrial fraction) was washed twice. The microsome fraction was obtained by centrifuging the remaining supernatant and washings in the Spinco preparative centrifuge at about 8°C, for one hour. The microsomal gel was not washed because it was well packed and difficult to resuspend. All three particulate fractions were resuspended and made up to a final volume of 10 ml. with ice-cold sucrose solution, and in the case of the microsomes, this required the use of the homogenizer. The final supernatant liquid, which contained the nongranular fraction of the cytoplasm was made up to a volume of 25 ml. with sucrose solution.

(b) Enzyme determination

Measurements were obtained in duplicate by the manometric method. The dilution of the cell fractions was governed by both the method of fractionation and the activity of the fractions. Each flask contained 0.5 ml. of diluted enzyme preparation, 4.0 ml. of 0.025 M bicarbonate solution and 0.5 ml. of 0.1 M substrate solution.

(c) Protein determinations

Protein nitrogens were determined by the micro Kjeldahl method and steam distillation. The ammonia released was taken up in 2 per cent boric acid containing bromcresol green and titrated with standard sulfuric acid.

The activities obtained when sarin was used as a substrate are

shown in Table IV. These results show a high degree of individual variation in the total specific activity. This variation is due mainly to differences between individual animals; some variation was contributed by deterioration in storage. It was noted that the loss in activity was quite high during the first 48 hours of storage. It was not always possible to perform the sarinase determinations at a fixed time after the animal was killed. In general the storage time was 16 hours. The maximum storage time was 64 hours, during which a loss in activity up to 30% was noted. A test made with fresh and stored tissue showed that the relationship between fractions was not affected by storage. In all cases the soluble fraction (supernatant) had the highest mean specific sarinase activity.

The microsome fraction usually had the next greatest specific activity, except rat kidney, mouse liver and monkey kidney. The nuclei in general had the lowest activity. In many animals the kidney nuclei activity was zero. Due to intact cells being precipitated with this fraction (87), the mean activity of the nuclear fraction is probably high. The activity of the mitochondrial fraction was a little higher than the nuclei. In no case was it zero.

When the sarinase activities for each fraction were listed as the per cent of the total activity in the sample used, the individual differences in total enzyme level were eliminated. Table V lists the sarinase activities expressed as percentages of the total activity in the sample used.

The activity of the supernatant fraction appears much higher when the data are treated in this manner; this fraction, however, is the

Table IV

Hydrolysis of sarin by Liver and Kidney Cell Fractions

Mean Specific Activities with Standard Errors

(µl. CO_2/30 min./g. N x 10^{-3})

Species and Organ	Number of Animals	Nuclei	Mitochondria	Microsomes	Supernatant	Total Homogenate
Rat liver	9	469 ± 152	1100 ± 253	1639 ± 246	2774 ± 256	1548 ± 235
Rat kidney	9	123 ± 70	627 ± 79	362 ± 50	1243 ± 111	918 ± 112
Mouse liver	6	545 ± 118	569 ± 107	531 ± 69	1915 ± 138	1357 ± 200
Mouse kidney	6	40 ± 14	104 ± 29	239 ± 57	572 ± 234	335 ± 178
Rabbit liver	4	612 ± 96	603 ± 76	1147 ± 134	1554 ± 255	1378 ± 123
Rabbit kidney	4	347 ± 156	486 ± 81	606 ± 79	920 ± 127	754 ± 156
Guinea pig liver	6	267 ± 31	256 ± 46	506 ± 42	611 ± 92	441 ± 51
Guinea pig kidney	6	133 ± 50	252 ± 68	363 ± 56	630 ± 59	423 ± 58
Monkey liver	5	62 ± 28	178 ± 27	269 ± 83	971 ± 274	794 ± 178
Monkey kidney	5	111 ± 20	352 ± 92	294 ± 36	833 ± 80	698 ± 120

Table V

Sarinase Levels in Cell Fractions

% of Total Activity (Sum of Fractions ± Standard Error)

Species and Organ	Nuclei	Mitochondria	Microsomes	Supernatant
Rat liver	2.2 ± 0.6	12.0 ± 2.2	20.6 ± 2.3	66.0 ± 2.7
Rat kidney	2.1 ± 0.7	13.6 ± 2.1	12.9 ± 1.1	69.8 ± 1.6
Mouse liver	5.8 ± 1.3	8.5 ± 1.2	15.6 ± 1.9	70.1 ± 2.1
Mouse kidney	3.1 ± 1.3	5.6 ± 1.5	15.1 ± 2.8	76.1 ± 4.1
Rabbit liver	8.1 ± 1.2	7.1 ± 1.4	25.4 ± 3.7	58.4 ± 5.4
Rabbit kidney	6.2 ± 3.3	9.3 ± 0.9	23.5 ± 2.7	60.0 ± 0.9
Guinea pig liver	9.2 ± 0.8	8.7 ± 1.1	28.5 ± 5.1	53.6 ± 4.8
Guinea pig kidney	7.7 ± 2.8	13.5 ± 1.9	20.5 ± 2.2	61.5 ± 2.6
Monkey liver	4.4 ± 2.0	15.9 ± 4.2	14.6 ± 5.1	65.0 ± 8.5
Monkey kidney	7.0 ± 1.9	16.8 ± 2.5	17.2 ± 1.6	59.1 ± 2.4

one which has the highest protein content.

The monkey liver preparations were also used to hydrolyze tabun in the same manner as sarin. To eliminate individual variations in total activity, the values for the fractions were compared on the basis of the per cent of total activity contributed by each fraction. Table VI shows this comparison.

It can be readily seen that no significant difference was found in the activity of any fraction towards the two substrates.

9. The Effect of Liver Enzyme on Toxicity

Because of the extreme toxicity of sarin and tabun, it is possible that variations in the amount of detoxication due to differences in hydrolyzing enzyme levels in the body may have a considerable effect on survival. To test this hypothesis, a series of experiments was carried out using sarin. The sarin was injected either intraperitoneally or subcutaneously into rabbits. The intraperitoneal route was chosen so that the injected sarin would pass through the liver before reaching the sites of effective cholinesterase inhibition; the portal circulation would be avoided by the subcutaneous injections. In both cases the sarinase of plasma could contribute to the total sarin detoxication. Sarinase levels in both liver and plasma were therefore determined in all the rabbits. In addition to these determinations, the plasma cholinesterase was measured. In order to differentiate between susceptible and resistant animals, the sarin was administered in small sublethal doses at regular intervals. Under these conditions, the most susceptible animals should die with the least number of doses. The possibility of the enzyme levels being increased after an initial dose was investigated by comparing the

Table VI

Comparison of Sarin and Tabun Hydrolysis by Monkey Liver

Cell Fraction	Activity (Expressed as percent of Total Activity ± Standard Error) Sarin Substrate	Tabun Substrate
Nuclei	4.4 ± 2.0	4.1 ± 1.8
Mitochondria	15.9 ± 4.2	16.2 ± 4.6
Microsomes	14.6 ± 5.1	15.3 ± 2.1
Supernatant	65.0 ± 8.5	64.4 ± 5.4

pooled liver and pooled plasma enzyme levels of normal rabbits and rabbits treated with one sublethal dose of sarin. As a further check on the effect of the injection on enzyme levels the variation encountered with normal animals was compared to that obtained with treated animals.

The rabbits used in this investigation were of mixed ancestry. The weights varied from 2 to 3.5 kg. The experiments were performed on nine groups of rabbits, five rabbits to a group. Although it was not found practical to confine the experiment to one sex, each group was of one sex only. Heparinized blood was obtained from the ear of each rabbit and centrifuged to obtain plasma, half of which was diluted, one part plasma to nineteen parts physiological saline. Each group was then injected with approximately one-third the rabbit $L.D._{50}$ dose of sarin (0.1 mg./kg. intraperitoneally and 0.02 mg./kg. subcutaneously) diluted in propylene glycol. Injections were made every fifteen minutes in the case of the intraperitoneally injected groups, and every half hour in the case of the subcuteneously injected groups. After death a sample of liver was removed, weighed, and homogenized first in a Waring blendor with thirty-nine parts of physiological saline and then with a teflon homogenizer to obtain uniform consistency. Preliminary experiments showed that sarinase is stable for at least thirty minutes at 55°C. Accordingly the homogenization was carried out at room temperature. To obtain the normal rabbit population variation of liver sarinase, a further ten rabbits (weight range as before) were sacrificed and samples of liver were removed, and processed as above. In addition, to check on any adaptive mechanism, five rabbits were injected with one dose (1/3 rabbit intraperitoneal $L.D._{50}$) of sarin and sacrificed after two hours, at which

time another five rabbits were sacrificed as controls. The liver samples from each group were pooled and the liver sarinase was determined. As a check on plasma enzyme adaptation a group of ten rabbits were bled before and after a L.D.$_{50}$ dose of sarin. Determination of plasma sarinase was made on blood from both groups.

Sarinase measurements were obtained in duplicate by the Warburg manometric method. The vessels contained 4.0 ml. of 0.025 M bicarbonate buffer, 0.5 ml. enzyme source (diluted liver or plasma) and 0.5 ml. of 0.1 M sarin (in the side arm). Rates of hydrolysis were determined on the basis of CO_2 production during the thirty minute period beginning two minutes after the addition of substrate. Cholinesterase measurements were obtained in a similar manner. In this case the vessels contained 4.0 ml. of 0.025 M bicarbonate buffer, 0.5 ml. undiluted plasma and 0.5 ml. of 0.02 M acetylcholine chloride (in the side arm). In all cases controls containing 0.5 ml. of physiological saline in the place of the enzyme preparation were used.

The sarinase determinations for the ten rabbits used to obtain normal population variations were carried out simultaneously as were the determinations used to compare treated with untreated rabbits. All other determinations were carried out in groups of five.

The sarinase levels in liver and plasma, the plasma cholinesterase levels and the doses required to kill each animal by intraperitoneal injection are shown in Table VII. A similar set of data for the subcutaneous injections is given in Table VIII.

The data for the intraperitoneal injections were then analysed to test for correlation between enzyme levels and doses required to kill.

Table VII

Comparison of Enzyme Levels and Susceptibility to Sarin

(Intraperitoneal Injections)

Rabbit Number	Liver sarinase µl.CO_2/30 min./ 5 mg.	Plasma sarinase µl.CO_2/30 min./ 20 mg.	Plasma cholinesterase µl.CO_2/30 min./500 mg.	Doses to Death
1	162	78	81	1
2	185	125	71	1
3	193	152	122	1
4	165	139	110	1
5	172	78	105	1
6	101	58	94	1
7	185	147	80	1
8	135	128	88	1
9	107	88	102	1
10	237	97	79	2
11	226	84	104	2
12	159	147	88	2
13	104	115	76	2
14	181	53	75	2
15	165	67	109	2
16	193	80	120	3
17	192	93	62	3
18	128	76	83	3
19	130	40	79	3
20	166	69	90	4
21	213	151	84	4
22	230	77	81	4
23	316	135	108	4
24	215	106	72	5
25	134	177	110	5
26	250	103	81	5
27	221	118	82	6
28	286	153	94	6
29	352	115	89	8
30	351	135	99	8

Table VIII

Comparison of Enzyme Levels and Susceptibility to Sarin

(Subcutaneous Injections)

Rabbit Number	Liver sarinase µl. CO_2/30 min./ 5 mg.	Plasma sarinase µl. CO_2/30 min./ 20 mg.	Plasma cholinesterase µl. CO_2/30 min./500 mg.	Doses to Death
1	126	78	47	2
2	16*	50	96	2
3	112	50	59	2
4	112	83	56	2
5	86	77	74	2
6	124	115	49	3
7	94	94	88	3
8	269	30	76	3
9	218	91	59	3
10	125	99	17	3
11	99	68	56	4
12	67	68	99	4
13	96	80	75	4
14	256	140	58	4
15	152	58	45	5

---

* Very anaemic animal.

No correlation was found to be present in the case of plasma sarinase and plasma cholinesterase. Liver sarinase levels compared with doses of sarin required to kill gave a correlation coefficient of 0.721, indicating high statistical significance. The liver enzyme data from Table VII were grouped according to doses required to kill and standard deviations were calculated. This information is shown in Table IX. Figure 18 is a graph plotted from these combined data and illustrates change of susceptibility with change in liver enzyme levels. The data from the subcutaneous injection experiments were examined statistically in the same manner. In this case no correlation was found between either liver or blood enzymes and doses required to kill.

The ten control rabbits, sacrificed to obtain normal population variations, gave liver sarinase levels as shown in Table X. A comparison of these figures with those given in Table VII and VIII shows that, for the control animals, a variance of 2876 is obtained and for the forty-five treated animals a variance of 5360. These figures give an F value of 1.9 (79) which is not significant at the 5% level. The group of five rabbits treated with 1/3 $L.D._{50}$ dose compared with the five untreated animals revealed a difference in liver sarinase level of only 5 units (treated = 187; untreated = 182), indicating no evidence of enzyme adaptation. The ten rabbits tested for plasma sarinase adaptation had a sarinase level of 145 before treatment and 136 after. The difference is within the experimental error.

III Discussion

The sarinase and tabunase activities of bovine plasma are

Table IX

Comparison of Liver Sarinase Levels with Susceptibility of Rabbits to Sarin

(Intraperitoneal Injections)

Number of Rabbits	Doses to Kill	Liver sarinase (μl.CO_2/30 min.) Mean	Standard deviation
9	1	156	33
6	2	179	49
4	3	160	37
4	4	231	63
3	5	200	92
2	6	253	46
2	8	352	2

Figure 18

Variation of resistance with levels of liver sarinase.

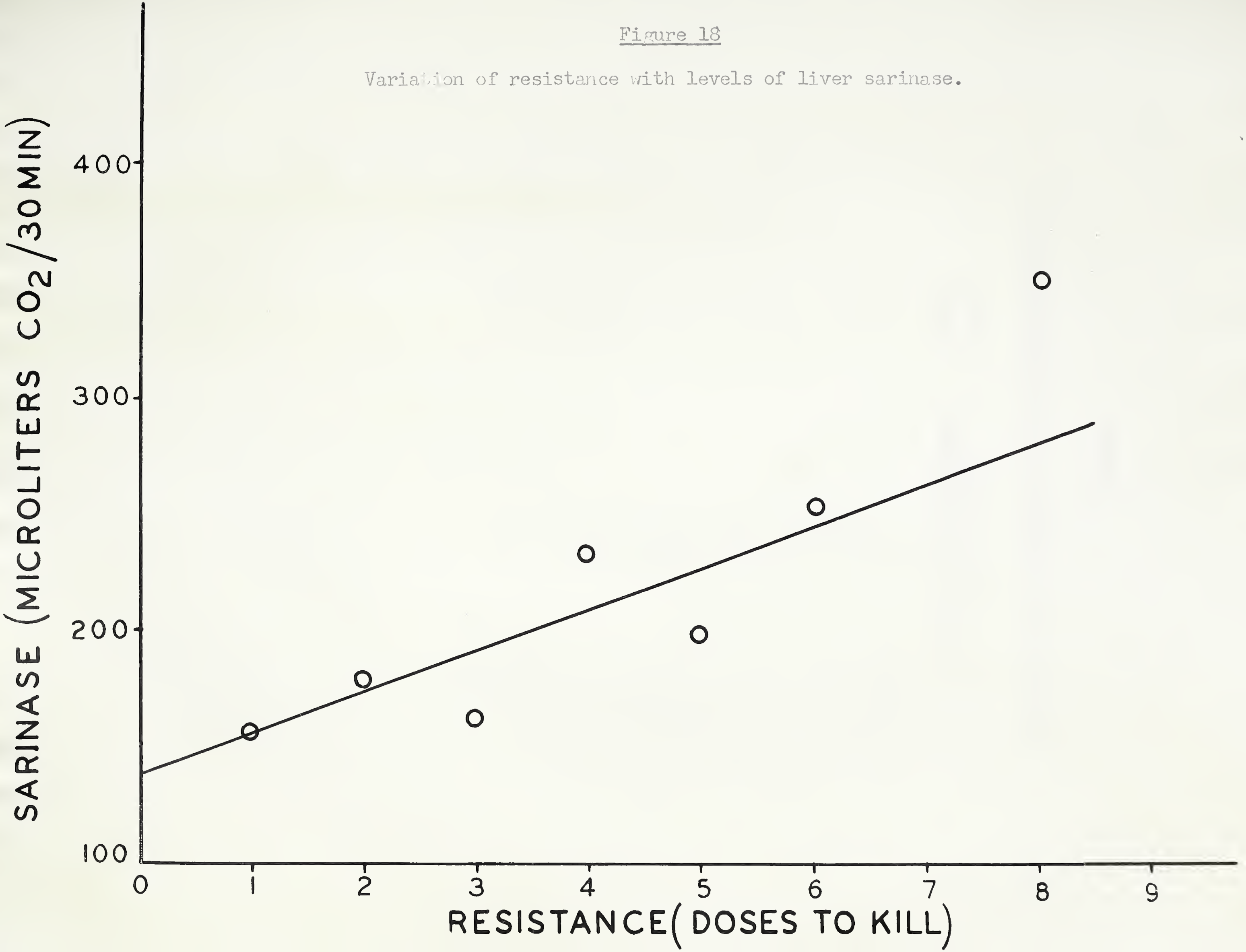


Table X

Normal Population Levels of Rabbit Liver Sarinase

Rabbit Number	Liver sarinase (μl.CO_2/30 min./5 mg.)
1	157
2	213
3	166
4	95
5	150
6	204
7	300
8	164
9	196
10	207

inactivated to the same extent when the plasma is exposed to varying degrees of heat (Figure 14), acidity and alkalinity (Figure 15). These results suggest that one enzyme is responsible for the hydrolysis of both substrates by plasma. This conclusion is supported by the results of the purification experiment. When the sarinase was purified 1200-fold by fractional precipitation of proteins from the plasma, the ratio of the rates of hydrolysis of sarin and tabun was not significantly changed.

The Michaelis constants for the enzyme in rat plasma are 7.0 x 10^{-3} M sarin and 7.6 x 10^{-3} tabun. These values are of the same order as those given by Augustinsson and Heimbürger (19) for the hydrolysis of tabun by the enzyme in horse plasma and by a purified enzyme from human blood.

The activation energies for the enzymic hydrolysis of sarin and tabun by plasma in the range between 5 and 50°C were both approximately 15,000 calories. Values for other enzymic reactions may vary from approximately 4,000 to 18,000 calories. The fact that similar energies of activation are obtained for two substrates with one enzyme preparation agrees with the supposition that both substrates are being hydrolyzed by the same enzyme in plasma.

The ratio of the rates of hydrolysis of sarin and tabun varies from one organ to another within the same species of animal (as well as from one species to another). This suggests that more than one kind of enzyme is present in the body. Similar variations in dialkylfluorophosphatase from different organs were demonstrated by Mounter and Chanutin (138). Comparison of the ratios of sarin hydrolysis to tabun hydrolysis

for different organs in several species shows that this ratio is 2 - 4 times higher in liver than in the plasma for all six species of animal investigated. Fractionation of liver cells from the monkey into the various components discloses that the ratio of sarin hydrolysis to tabun hydrolysis is similar in each cell fraction. This result suggests that the liver contains only one enzyme which catalyzes the hydrolysis of sarin and tabun; as noted above, this enzyme is different from the plasma enzyme.

There appear to be small differences in the distribution of sarinase in liver and kidney cell fractions from one species to another. However, these differences are not related to the differences in resistance of the species to sarin poisoning.

Calculations based on the K_m value for plasma sarinase indicated that this enzyme could destroy 50% of the sarin injected into the blood stream within one minute (see page 36). However, there is no correlation between the plasma sarinase level and the toxicity of sarin from one animal to another; nor was there any correlation between the liver sarinase level and the toxicity of sarin when this inhibitor was injected subcutaneously. This finding was surprising but could be explained if the sarin combines with the essential cholinesterase much more rapidly than it is hydrolyzed by the sarinase. This possibility will be investigated below (see page 88).

On the other hand, when the sarin is injected intraperitoneally so that it passes through the liver before entering the general circulation, a correlation between the level of liver sarinase and the resistance of the animal to sarin poisoning is observed. This suggests that the detoxic-

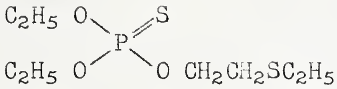
ation of sarin by liver sarinase is responsible for the fact that the lethal dose of sarin injected intraperitoneally is much higher than the subcutaneous lethal dose.

When one considers the detoxication carried out by other organs as shown in Table II, we can see that the main sites of destruction are likely to be the liver, kidney, intestinal mucosa and spleen. Thus it is likely that should inhibitors of the hydrolyzable type be ingested with food i.e. as insecticide residues in plants, the toxicity could be affected in the intestines during absorption. The oral toxicity of sarin is ,in fact,known to be less than the intraperitoneal toxicity. This result appears to be comparable to the effect of liver sarinase,as mentioned above. That is to say, the sarinase of liver and intestinal mucosa will detoxify an appreciable portion of the sarin when this compound must pass through these organs before reaching the general circulation.

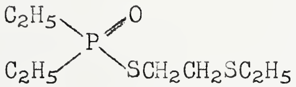
PART 2 -- STUDIES ON THE DETOXICATION OF ISOSYSTOX

I Introduction

Isosystox is one of the two active components of the systemic insecticide Systox, the formulae for which are as follows:



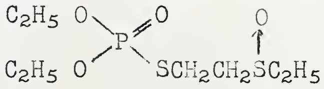
O, O-diethyl O-ethyl-2-mercaptoethyl phosphorothionate (systox)



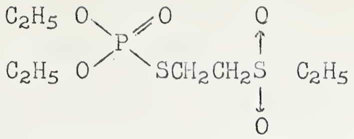
O, O-diethyl S-ethyl-2-mercaptoethyl phosphorothiolate (isosystox)

The ratio of thiono to thiol isomer in Systox is approximately 2:1.

Systox was first synthesized by Schrader in 1948 (155). Its systemic action is due to the metabolic products that are formed in biological systems as well as to the unchanged isomers. These metabolites, produced by enzymatic oxidations in plant and animal tissue, are more potent inhibitors of cholinesterase than the parent compounds. In the case of isosystox, March, Metcalf, Fukuto and Maxon (122) have shown that it is converted in the mouse first to the sulfoxide



and then to the sulfone



They found that the concentration of isosystox and its sulfoxide and sulfone required to inhibit a given amount of cholinesterase in a given time was as follows:

Isosystox	3.5×10^{-6} M
Isosystox sulfoxide	1.5×10^{-6} M
Isosystox sulfone	6.0×10^{-7} M

All three compounds were found to be hydrolyzable at the P-S bond.

The toxic action of isosystox is due to its own anticholinesterase action and also to that of its metabolites, the sulfoxide and sulfone. The reason for its low toxicity does not seem to be detoxication by hydrolysis. March and co-workers (122) recovered toxic products in mouse liver and urine several hours after oral administration of isosystox. Therefore, in relation to sarin and tabun, the life of isosystox and its toxic metabolites in the animal is extremely long.

II Experimental and Results

1. Chromatographic experiments

Since March and associates (122) suggest that liver is the main site of degradation of isosystox, it was decided to check the *in vitro* detoxication carried out by rat liver. As a preliminary experiment, the oxidation of isosystox was performed with rat liver homogenate. The products formed were compared with the sulfoxide and sulfone obtained with

peroxide and permanganate oxidation (73).

Peroxide oxidation was carried out by dissolving a 0.5 ml. sample of isosystox in 0.5 ml. of acetone and adding 1.0 ml. of 30% hydrogen peroxide. The mixture was allowed to stand for three hours. Water was then added and the products were extracted with chloroform. The chloroform solution was washed with 10% aqueous ferrous sulfate and dried over anhydrous sodium sulfate. The chloroform was then removed under vacuum. Approximately 50% of the isosystox had been converted to sulfoxide during this period (Figure 19). Fukuto, Metcalf, March and Maxon (73) found complete conversion by this time.

Permanganate oxidation was performed on a 5 g. sample of isosystox dissolved in 10 ml. of glacial acetic acid. To this was added 5 g. of potassium permanganate dissolved in 100 ml. of water. This solution was stirred for an hour, was decolourized by the addition of sodium bisulfite and extracted with three 10 ml. portions of chloroform. The chloroform extract was dried over anhydrous sodium sulfate and the chloroform was removed under vacuum. A chromatogram of the product, shown in Figure 20, revealed that a considerable quantity of the isosystox remained unchanged. However an appreciable amount of sulfoxide and sulfone was found. The two slow moving spots have not been identified. The R_f value of the isosystox spot in this case is 0.24 as compared with 0.40 obtained previously (see page 18). This is because the chromatogram was developed on Whatman No. 4 paper; a filter paper which gives good definition but very slow flow. A control strip of isosystox gave a spot with R_f 0.24 on this paper.

Oxidation by Liver Homogenates. A 0.5 ml. sample of isosystox

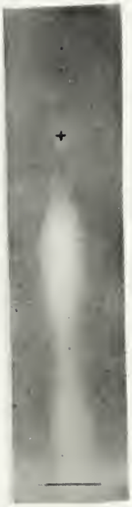


Figure 19

Effect of peroxide oxidation of isosystox. (Whatman #3 MM paper.)

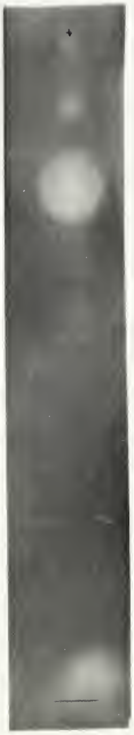


Figure 20

Effect of permanganate oxidation of isosystox. (Whatman #4 paper.)

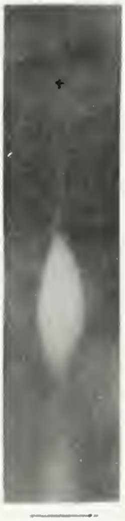


Figure 21

Effect of incubation of isosystox with liver homogenate. (Whatman #3 MM paper.)

was shaken with 10 grams of rat liver homogenate for four hours at 37°C. The reaction mixture was extracted with three 10 ml. portions of chloroform and dried with anhydrous sodium sulfate. The chloroform was evaporated and a sample of the residue was chromatographed. A small amount of sulfoxide-sulfone was seen to be present on the chromatogram (Figure 21).

Oxidation by liver slices and liver brei* was carried out in a similar manner. The result obtained was the same as that produced with liver homogenate, i.e. more of the material remained as isosystox than was oxidized. The rather poor oxidations obtained suggested that perhaps the oxidative system required additional cofactors. Accordingly incubations were performed with 10 grams of liver slices plus 10 ml. of potassium phosphate buffer pH 7.4 (0.01 M) containing cytochrome c (0.0075 M), magnesium chloride (0.006 M), adenosine triphosphate (ATP) (0.001 M), sucrose (0.25 M), succinic acid (0.01 M) and diphosphopyridine nucleotide (DPN) (0.0005 M).

A chromatogram of a chloroform extraction from the incubation mixture showed no increase in oxidation products. Tests using uridine monophosphate (0.001 M) or guanosine diphosphate (0.001 M) in place of adenosine triphosphate and the addition of flavine adenine dinucleotide (0.005 M) were also negative.

2. Warburg Experiments

The destruction of isosystox by liver slices was followed using the manometric technique. The chromatographic method does not show inactive products because they do not inhibit the cholinesterase used to localize the spots. Ten grams of rat liver slices were incubated

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* See page 75 for methods of production.

in 20 ml. of a medium which contained: potassium phosphate buffer pH 7.4 (0.01 M), cytochrome c (0.0075 M), ATP (0.001 M), succinic acid (0.005 M), DPN (0.0005 M) and isosystox (4.4×10^{-5} M). At the time intervals recorded in Table XI, 10 ml. samples of the medium were withdrawn and extracted with three 10 ml. portions of chloroform. The chloroform was then evaporated under vacuum and the residue taken up in 1 ml. of normal saline. A 0.5 ml. sample of this was pipetted into the main compartment of a Warburg flask which contained 0.5 ml. of purified bovine erythrocyte cholinesterase (diluted so that a control without isosystox produced approximately 200 µl. CO_2/30 min.), 0.34 ml. (in the side arm) of 0.44 M acetyl β methylcholine chloride (Mecholyl) solution (final concentration 0.03 M) and 3.66 ml. of 0.025 M bicarbonate buffer. The mixture was allowed to incubate for 30 minutes to ensure inhibition of cholinesterase before the Mecholyl was tipped in. Thus the relative amount of inhibitor present at the time the sample was removed from the liver slices was determined.

Although a considerable amount of inhibitor disappears within the first minute, there seems to be little further destruction up to four hours. In view of the chromatographic findings, this suggests that under these conditions the amount of oxidation to non-toxic material is small. The reason for the initial rapid disappearance of isosystox will be considered below.

The destruction of isosystox by liver brei and liver homogenates was also followed manometrically. Liver brei was produced by forcing rat liver through the end of a hypodermic syringe (with no needle attached). The detoxifying activity of this material was compared with that of a

Table XI

Cholinesterase Activity Remaining after a 30 Minute Incubation with Isosystox Recovered from a Rat Liver Slice Incubation Mixture at the Times Recorded

Time (min.)	% of Control Activity *
0	6
1	58
2	56
4	60
8	62
15	59
30	59
60	53
120	59
180	64
240	64

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* The control flasks contained purified bovine erythrocyte cholinesterase, buffer and Mecholyl (see page 75). Thus this column represents the destruction of isosystox; a low figure shows that considerable isosystox is present i.e. the purified cholinesterase is inhibited.

liver homogenate produced in a Potter homogenizer in the following manner:

0.5 g. of brei was weighed into Warburg flasks and 0.5 ml. of 8.8×10^{-6} M isosystox in isotonic sucrose plus a further 0.5 ml. of isotonic sucrose were added. In the case of homogenate 5 g. of liver were homogenized with 5 ml. of isotonic saline. 1 ml. of this was pipetted into Warburg flasks and 0.5 ml. of 8.8×10^{-6} M isosystox in isotonic saline was added. The mixtures were allowed to incubate for (a) 15 minutes and (b) 30 minutes. At the end of the incubation time 2.66 ml. of 0.025 M bicarbonate solution and 0.5 ml. of purified bovine erythrocyte cholinesterase were added to the main compartment and 0.34 ml. of 0.44 M Mecholyl solution to the side arm. The flasks were then allowed to incubate for 30 minutes before tipping. Manometric assay of cholinesterase activity was carried out in the usual manner. The results are shown in Table XII. The amount of isosystox destroyed by the brei was not significantly different from the amount destroyed by the homogenate.

The detoxifying action of rat liver homogenate in which the cholinesterase had been inhibited was examined to see if cholinesterase inhibition could account for the initial rapid disappearance of isosystox shown in Table XI. A sample of rat liver was homogenized in a Potter homogenizer with an equal quantity of isotonic saline. To this was added a sufficient quantity of sarin (0.0015 mg./ml.) to completely inhibit the cholinesterase. This sample was left for 30 minutes at room temperature so that the excess sarin was hydrolyzed. A portion of the reaction mixture was added to purified bovine erythrocyte cholinesterase to confirm the absence of sarin. One ml. of the homogenate was then transferred to a Warburg flask and 0.5 ml. of 1.76×10^{-5} molar isosystox was

Table XII

Activity of Purified Cholinesterase Inhibited by the Isosystox Remaining after the Incubation of Isosystox with Rat Liver Brei and Rat Liver Homogenate

Incubation Time Isosystox + Liver	% Control Activity Brei	 Homogenate
15 min	10	12
30 min	9	10

added. The mixture was incubated for 30 minutes to permit the destruction of isosystox then 0.5 ml. of purified bovine erythrocyte cholinesterase solution, 0.34 ml. of mecholyl solution (in the side arm) and 2.66 ml. of bicarbonate solution were added to measure the amount of isosystox remaining.

This amount was compared with the isosystox which remained after incubation with normal rat liver. The results are shown in Table XIII, and indicate that the initial rapid _in vitro_ uptake of isosystox in liver homogenate is the result of its combination with esterases.

As a further check on the above conclusion the cholinesterase content of liver and kidney cell fractions was compared with their ability to destroy isosystox. A 1.0 g. sample of rat liver was fractionated and diluted as described previously (page 52) into nuclei (and intact cells), mitochondria, microsomes and non-granular supernatant fraction. A 0.5 ml. sample of each fraction was tested for cholinesterase activity manometrically using 0.036 M acetylcholine chloride as substrate. A 1.0 ml. sample of each fraction was incubated with 0.5 ml. of 4.4×10^{-5} M isosystox for 30 minutes then tested for residual isosystox activity with bovine erythrocyte cholinesterase and mecholyl as before.

The above sequence was repeated using rat kidney cell fractions. In this case the cholinesterase activity was measured with 2.0 ml. samples. Table XIV lists the results of these experiments and shows that the isosystox "destruction" follows reasonably well the cholinesterase activity of kidney and liver cell fractions.

3. _Excretion of Isosystox in Urine_

The amount of an anticholinesterase excreted by the kidneys will,

Table XIII

Comparison of the Detoxifying Activity of Normal Rat Liver With Liver in which the Cholinesterase had been Inhibited

Activity (% of Control)		
Sarin treated Liver + Isosystox	Untreated Liver + Isosystox	Isosystox Alone
13	85	9

Table XIV

Comparison of Cholinesterase Activity and Isosystox Destroying Capacity of Rat Liver and Rat Kidney Cell Fractions

	Tissue			
	Liver		Kidney	
Cell Fraction	Cholinesterase Activity of liver (μl.CO_2/30 min.)	Isosystox Destruction (Erythrocyte Cholinesterase Activity Remaining After Incubation) (μl. CO_2/30 min.)	Cholinesterase Activity of kidney (μl.CO_2/30 min.)	Isosystox Destruction (Erythrocyte Cholinesterase Activity Remaining After Incubation) (μl. CO_2/30 min.)
Rat 1				
Nuclei	56	74	77	13
Mitochondria	105	92	80	12
Microsomes	113	99	106	37
Supernatant	51	79	99	26
Rat 2				
Nuclei	49	64	64	22
Mitochondria	102	98	68	23
Microsomes	128	109	94	54
Supernatant	83	86	80	34

of course, influence the toxicity of the compound. If an inhibitor is slow in its reaction with cholinesterase, there may be time for considerable excretion of the inhibitor from the body before a toxic degree of inhibition takes place.

It is improbable that sarin or tabun would appear unchanged in urine since these compounds are rapidly hydrolyzed by sarinase. However isosystox, or its inhibitory oxidation products, have been shown by March and associates (122) to be excreted in urine. The total amount recovered from the urine was 4 - 5% of an orally administered dose of 24 mg. isosystox/kg. in the mouse.

March and co-workers (122) measured the excretion of these compounds by means of a radioactive tracer which was not available for the present series of experiments. However, if a method of detection for anticholinesterases in urine were sensitive enough, it could be used to estimate the amount excreted after injection of a sub-lethal dose of non-radioactive isosystox. Since the slow absorption of the inhibitor into the blood stream after oral administration might influence the amount excreted in the urine, an attempt was made to confirm the excretion of isosystox in the urine after injection, rather than oral administration, of isosystox.

A rat was anaesthetized with "Dial" and was prepared for the experiment by exposing the bladder and inserting a plastic catheter. The wound was sewed up leaving the drainage catheter free. The animal was positioned belly down on a platform with the catheter inserted through a hole in the platform so that it drained into collecting tubes which were changed every twenty minutes. After a sample of normal urine

had been collected, the rat was given a 3 mg./kg. dose of isosystox.

The urine samples were tested by the Michel electrometric method (133) using 1,0 ml. of horse serum, diluted 1:10, 0.02 ml. of urine, 1.0 ml. of Michel No. 2 buffer and 0.2 ml. of 0.165 M acetylcholine chloride solution. This method is sensitive to at least 0.2 µg. of inhibitor.

Two experiments were carried out. Urine samples were collected at twenty minute intervals for 5.3 hours after intravenous injection of isosystox in the first experiment and for 2.3 hours after subcutaneous injection of isosystox in the second. No appreciable amount of inhibitor could be detected in any of the urine samples. Considering the sensitivity of this method and the volume of urine excreted, this means that less than 2% of the injected dose was excreted in the urine each hour.

4. Toxicity Determinations

The marked difference in the toxicities of sarin after subcutaneous and intraperitoneal injections was mentioned above in connection with the influence of liver sarinase on the toxicity of sarin to rabbits. If an appreciable amount of an anticholinesterase is detoxified in liver, then the toxicity of this compound should be much smaller after intraperitoneal than after subcutaneous or intravenous injections. This criterion could be used to determine whether or not an appreciable detoxication of isosystox occurs in the liver. For purposes of comparison, the toxicities of sarin and tabun were determined in the same manner as for isosystox.

The intravenous, subcutaneous and intraperitoneal toxicities of sarin, tabun and isosystox were determined in male mice from a heterogeneous strain. Ten mice were injected at each dose level. Deaths were recorded

24 hours after injection. The volume of material injected was 0.1 ml. The intravenous injections were made into a tail vein in ten seconds. Table XV records the results of these injections.

The L.D.$_{50}$ values, obtained from these data by the method of Finney (68), are shown in Table XVI. The toxicity of isosystox is not significantly affected by the route of administration. On the other hand, marked differences are observed with sarin and tabun which can be ascribed to the detoxifying effect of sarinase on these compounds. For example, the decrease in toxicity when the inhibitor is administered intraperitoneally in place of subcutaneously amounts to 6% for isosystox (not statistically significant), 208% for sarin and 470% for tabun (Table XVI). This result suggests that the detoxication of isosystox in the liver is not rapid enough to affect the toxicity.

III Discussion

March and co-workers (122) recovered oxidation products from the liver of mice. The present experiments confirm that some oxidation occurs *in vitro* but this process did not appear to be rapid. Oxidation does not account for the major portion of the isosystox which disappears from the reaction mixture with liver *in vitro*. There appears to be a rapid initial uptake of isosystox by liver cells which is virtually eliminated after liver esterases have been inhibited with sarin. The distribution of cholinesterase in liver cells follows reasonably well the distribution of the isosystox-combining power. These results suggest that non-essential cholinesterase and other esterases combine with considerable amounts of isosystox.

Table XV

Data for Determining the Toxicities of Sarin, Tabun and Isosystox Injected Intravenously, Subcutaneously and Intraperitoneally

Number of deaths after 24 hours

Route of Administration	Dose (µg./kg.)	Sarin Deaths	Dose (µg./kg.)	Tabun Deaths	Dose (mg./kg.)	Isosystox Deaths
intravenous	77	0/10	116	0/10	5.2	0/10
	116	3/10	154	3/10	6.9	2/10
	154	4/10	231	7/10	8.2	5/10
	231	8/10	308	10/10	10.3	9/10
	308	10/10			12.1	10/10
subcutaneous	110	0/10	174	0/10	5.3	0/10
	165	2/10	261	2/10	7.1	0/10
	220	6/10	348	5/10	8.8	3/10
	330	8/10	422	9/10	10.6	7/10
	440	10/10	696	10/10	12.4	10/10
intraperitoneal	226	0/10	466	0/10	5.3	0/10
	339	3/10	699	0/10	7.1	3/10
	452	5/10	932	6/10	8.8	6/10
	678	7/10	1398	6/10	10.6	9/10
	904	10/10	1864	10/10	12.4	10/10

Table XVI

Toxicities of Sarin, Tabun and Isosystox Injected Intravenously, Subcutaneously and Intraperitoneally

Inhibitor	Route of Administration	L.D.$_{50}$ (mg./kg.)	95% confidence limits of L.D.$_{50}$ value (mg./kg.)
isosystox	intravenous	7.66	(6.9 - 8.5)
	subcutaneous	9.52	(8.9 - 10.3)
	intraperitoneal	8.12	(7.4 - 8.9)
sarin	intravenous	0.154	(0.135 - 0.185)
	subcutaneous	0.220	(0.188 - 0.257)
	intraperitoneal	0.457	(0.397 - 0.569)
tabun	intravenous	0.188	(0.165 - 0.216)
	subcutaneous	0.331	(0.289 - 0.379)
	intraperitoneal	1.072	(0.906 - 1.266)

Subsequent experiments showed that the amounts of isosystox excreted in the urine must be very small. However, the method used was not sensitive enough to detect the excretion of less than 2% of the total dose of isosystox per hour. March and associates (122) recovered a total of 1 - 5% from the urine within 4 hours and 4 - 5% from the urine within 24 hours after oral administration of isosystox. In their experiments, no significant amount of isosystox or breakdown products could be detected in the faeces. These figures again indicate that little of the active anticholinesterase is lost by excretion. Isosystox appears therefore to be a cholinesterase inhibitor which is not detoxified rapidly and which is not lost from the animal by excretory processes.

Toxicity measurements confirmed the conclusion that there is little destruction of isosystox in the body, apart from the "detoxication" due to reaction with esterases other than the essential cholinesterase. (This latter reaction will be considered in the next section.) The toxicity of isosystox was essentially the same regardless of the route of its administration. In contrast, sarin and tabun were shown to be much less toxic when injected intraperitoneally than when injected intravenously or subcutaneously.

Table XVI shows that isosystox is less toxic than sarin and tabun despite the fact that it is not rapidly detoxified. The above experiments have failed to disclose the reason for its low toxicity. This aspect of the problem will be considered in the next section.

PART 3 -- CHOLINESTERASE INHIBITION BY SARIN, TABUN AND ISOSYSTOX

I Introduction

Relation between inhibition rates and toxicity has been mentioned in the General Introduction. In this section the rates of true and pseudo cholinesterase inhibition by sarin, tabun and isosystox are reported. In addition the activation energies are obtained for the inhibitory reaction with isosystox. Inhibitors were used in concentrations greater than the enzyme concentration in order to ensure pseudomonomolecular reactions.

II Experimental and Results

1. Rate of Inhibition

The inhibition of cholinesterase was followed at 25°C. For studies of this type it is necessary to ensure that both mixing and incubation are carried out at a constant temperature. A unit designed for this purpose is shown in Figure 22. The flask was of the type which could be fitted to a Warburg manometer, be immersed in a water bath and be gassed with a 95% N_2 - 5% CO_2 mixture. The syringes were special tuberculin syringes with rubber plungers*. The needles were 1 1/2 inch long and gauge No. 20. The rubber caps were of the type normally fitted to Beckman pH meter reference electrodes. The unit was immersed in a constant temperature bath so that the liquid in the syringes was covered and was shaken during incubation.

The main body of the flask contained 1 ml. of horse serum or

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* Obtainable from Becton Dickinson Co.

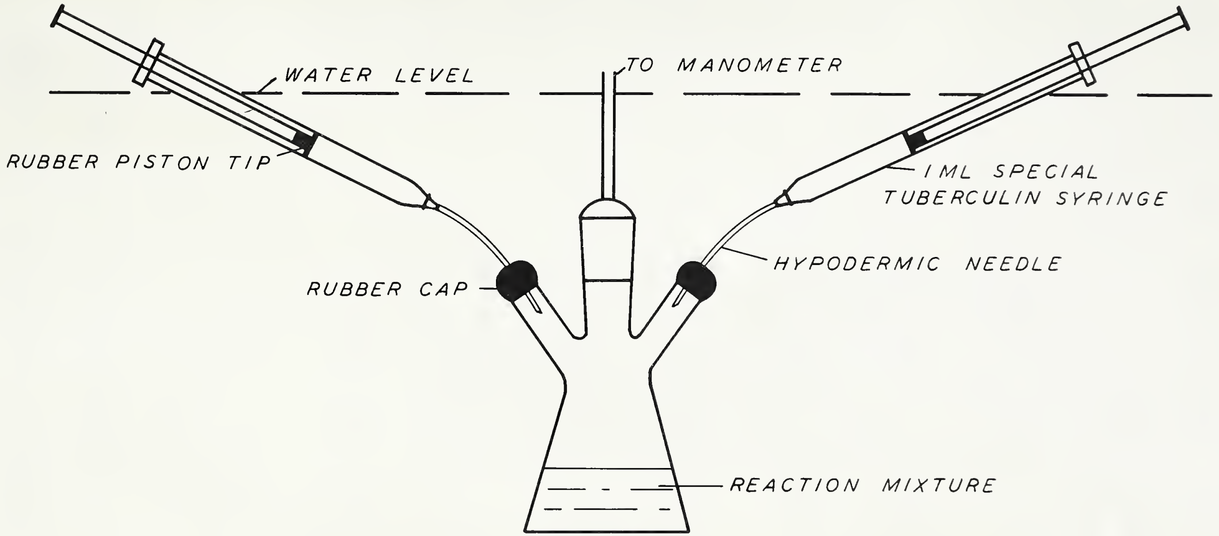

Figure 22

Diagram of reaction unit used
for measuring rates of cholinesterase inhibition.

bovine erythrocyte cholinesterase solution, 2.25 ml. of 0.025 M bicarbonate buffer. One syringe contained 1 ml. of either sarin, tabun or isosystox at concentrations which gave measurable inhibition rates; the other syringe contained either 0.75 ml. of 0.36 M acetylcholine chloride solution (for experiments with the non-specific cholinesterase of horse serum) or 0.75 ml. of 0.18 M* acetylcholine chloride (for determinations with the purified specific cholinesterase from bovine erythrocytes).

Gassing was accomplished by replacing one hypodermic syringe with a syringe barrel only. The assembly was removed from the water bath long enough to fit a syringe containing a reactant and then it was returned to the bath for temperature equilibration.

The inhibition reaction commenced when the plunger of the syringe containing inhibitor was pushed as rapidly as possible. Usually about 0.2 seconds was required for this operation. The reaction was stopped by injecting acetylcholine chloride solution in a similar manner (32, 39). The jet of liquid produced by pushing the syringe plungers provided thorough and practically instantaneous mixing.

After the injection of the acetylcholine chloride the flask was transferred to a Warburg bath where measurement of cholinesterase activity was carried out at 37°C in the usual manner. A thermal barometer flask was transferred from the incubation bath to the 37° bath at the same time as the reaction vessel.

The results of the six inhibition reactions are shown in graphical form in Figures 23 to 25 inclusive. Bimolecular reaction con-

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* This is above optimum concentration recommended for use with true cholinesterase but is that which gave the best straight line release of gas under the experimental conditions used.

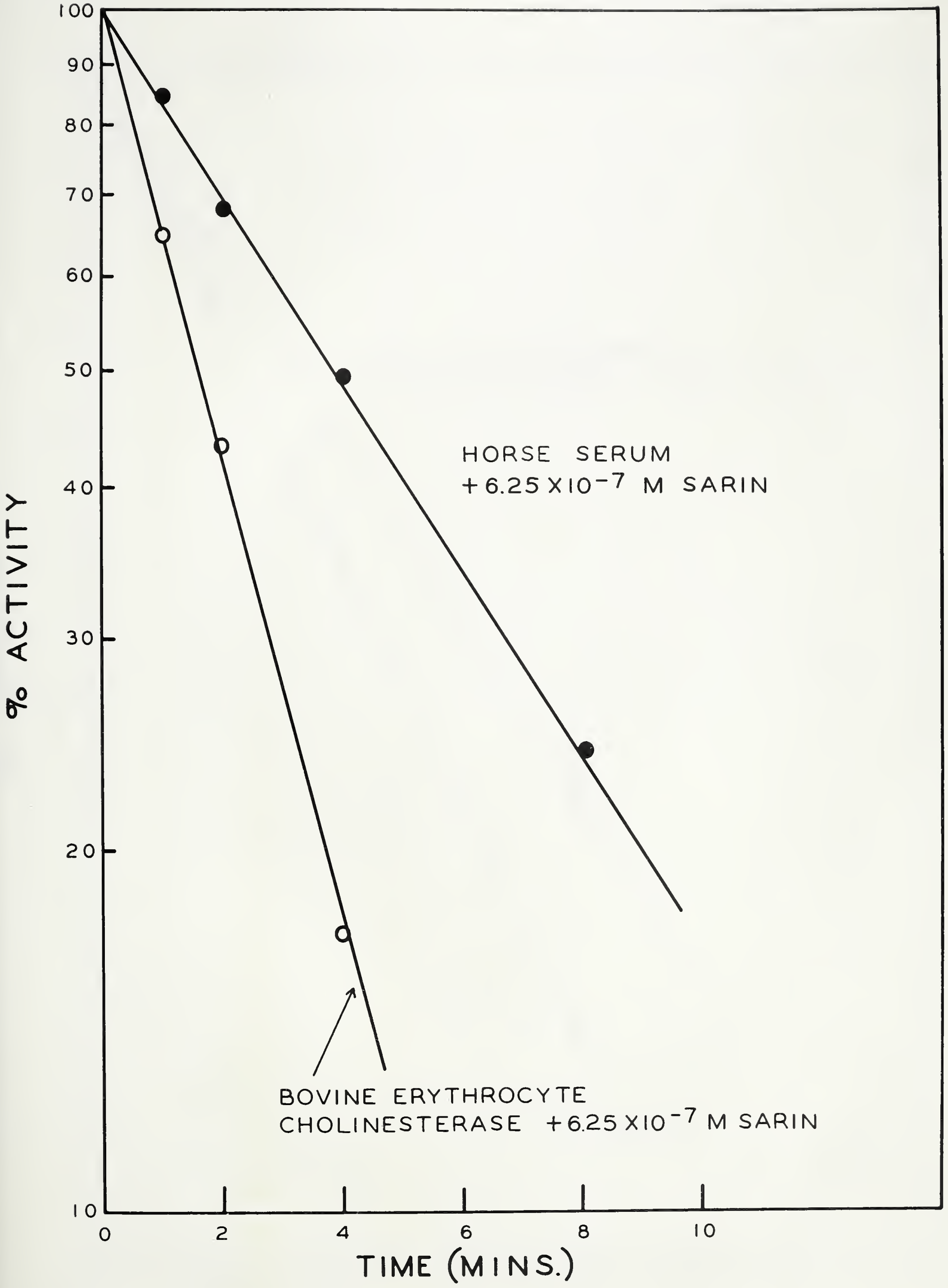

Figure 23

Inhibition of horse serum and purified bovine erythrocyte cholinesterases by sarin.

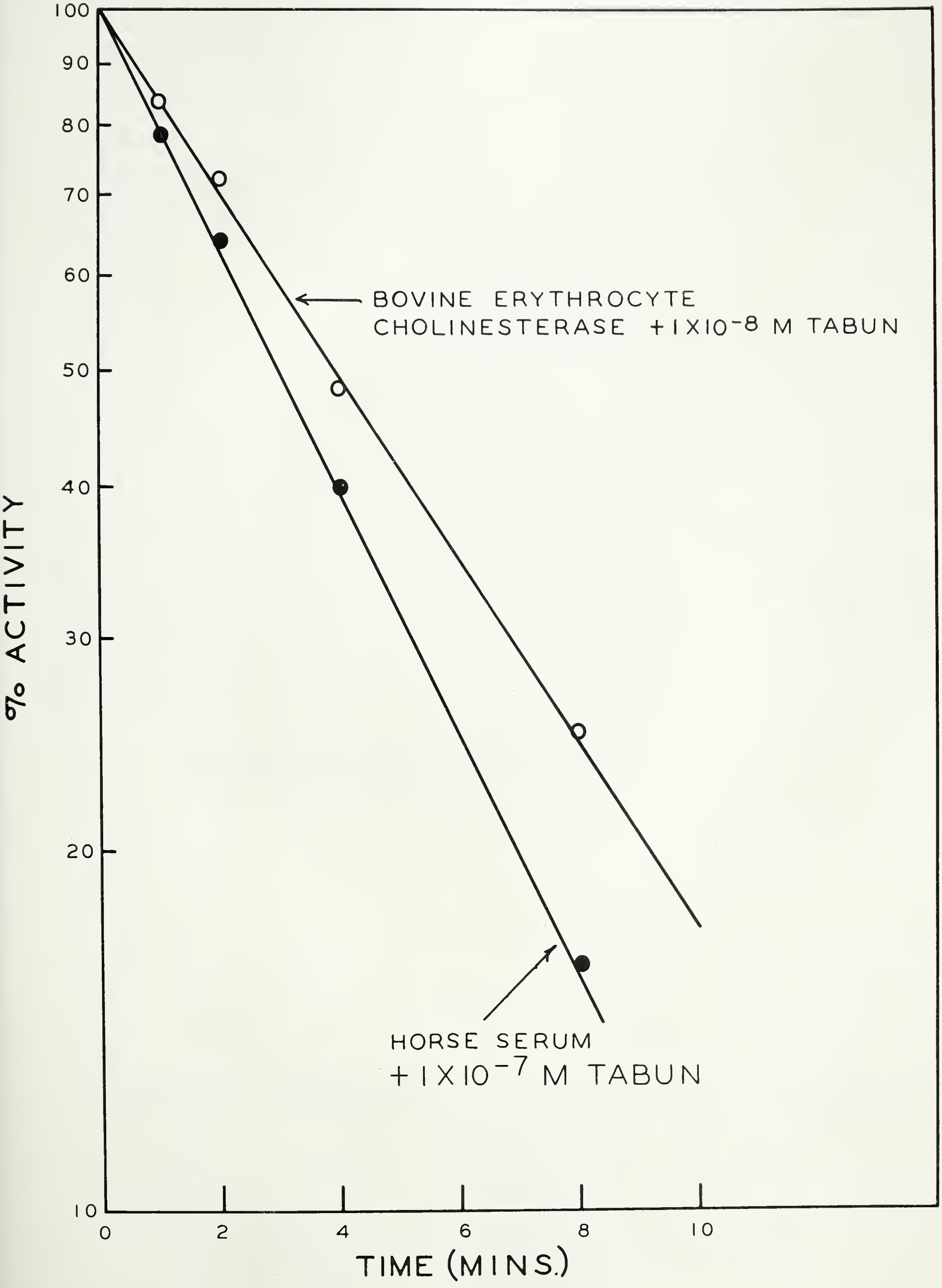

Figure 24

Inhibition of horse serum and purified bovine erythrocyte cholinesterases by tabun.

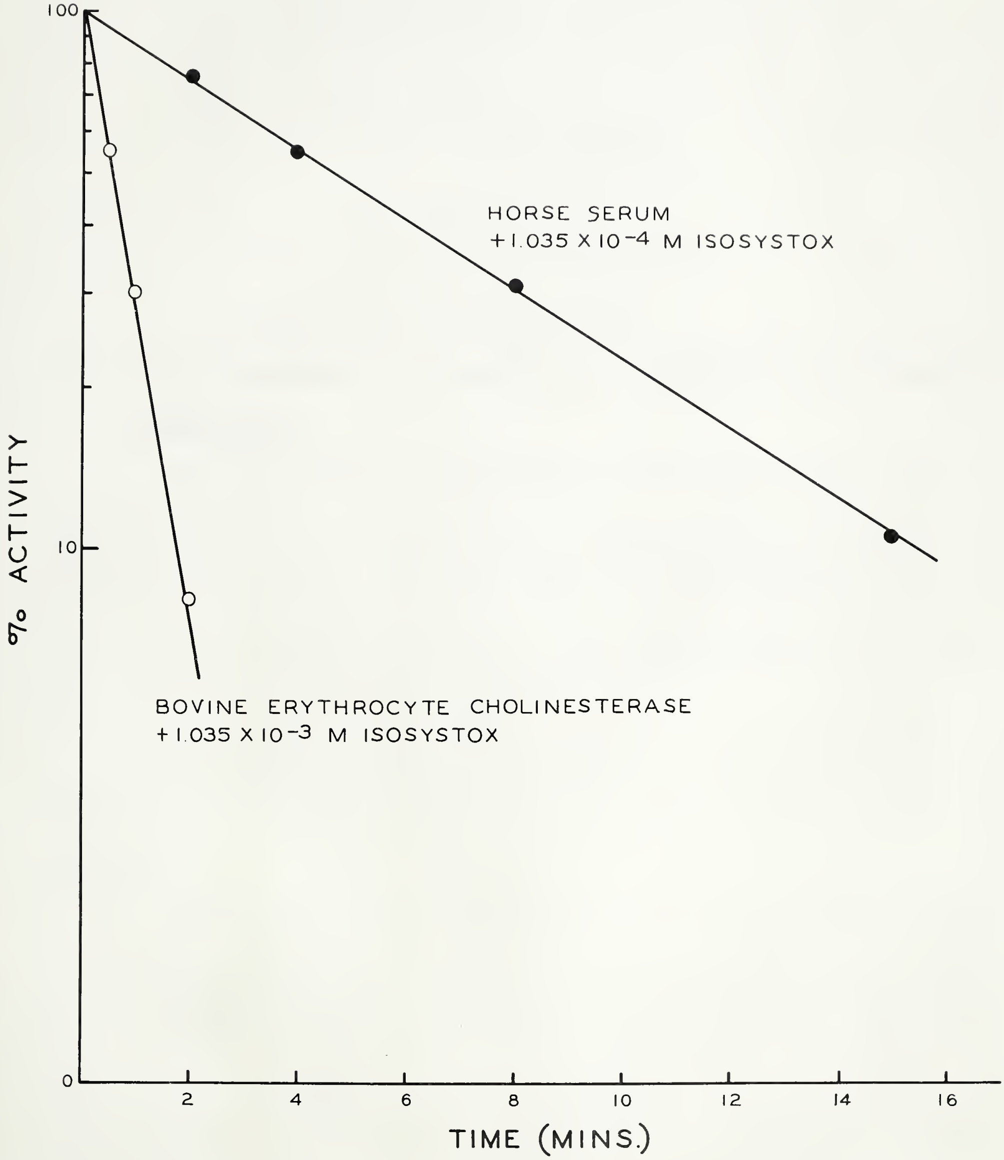

Figure 25

Inhibition of horse serum and purified bovine erythrocyte cholinesterases by isosystox.,

stants were calculated from the half lives obtained according to the formula $t_{\frac{1}{2}}\,b = \frac{.69}{k}$ *.

The constants for inhibition at 25°C are as follows:

sarin + non-specific cholinesterase	1.3×10^7 litre mole^{-1}min^{-1}
sarin + specific cholinesterase	6.9×10^7 litre mole^{-1}min^{-1}
tabun + non-specific cholinesterase	1.3×10^6 litre mole^{-1}min^{-1}
tabun + specific cholinesterase	1.7×10^7 litre mole^{-1}min^{-1}
isosystox + non-specific cholinesterase	1.1×10^4 litre mole^{-1}min^{-1}
isosystox + specific cholinesterase	1.3×10^3 litre mole^{-1}min^{-1}

2. Activation Energy for the Inhibition of Horse Serum Cholinesterase by Isosystox

The experiments were performed in the same manner as those described above in the previous section, except that the temperature of incubation was varied. Figure 26 shows the results obtained with the non-specific cholinesterase from horse serum. Reaction constants calculated from this data are listed in Table XVII.

The constants were plotted against the reciprocal of the absolute temperature (Figure 27) and an activation energy of 13500 cal./mole was obtained.

Similar experiments were carried out on the specific cholinesterase from bovine erythrocytes. Figure 28 shows the results obtained. Reaction constants calculated from the data are listed in Table XVIII. These reaction constants are plotted against the reciprocal of the absolute temperature in Figure 29. From this graph the activation energy was

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* It will be noticed that the b term is retained in the pseudomonomolecular reaction, this is because the equation for these conditions is

$$K = \frac{1}{tb} \ln \frac{a}{a-x} \quad \text{(units = litre mole}^{-1}\text{min}^{-1}\text{)}.$$

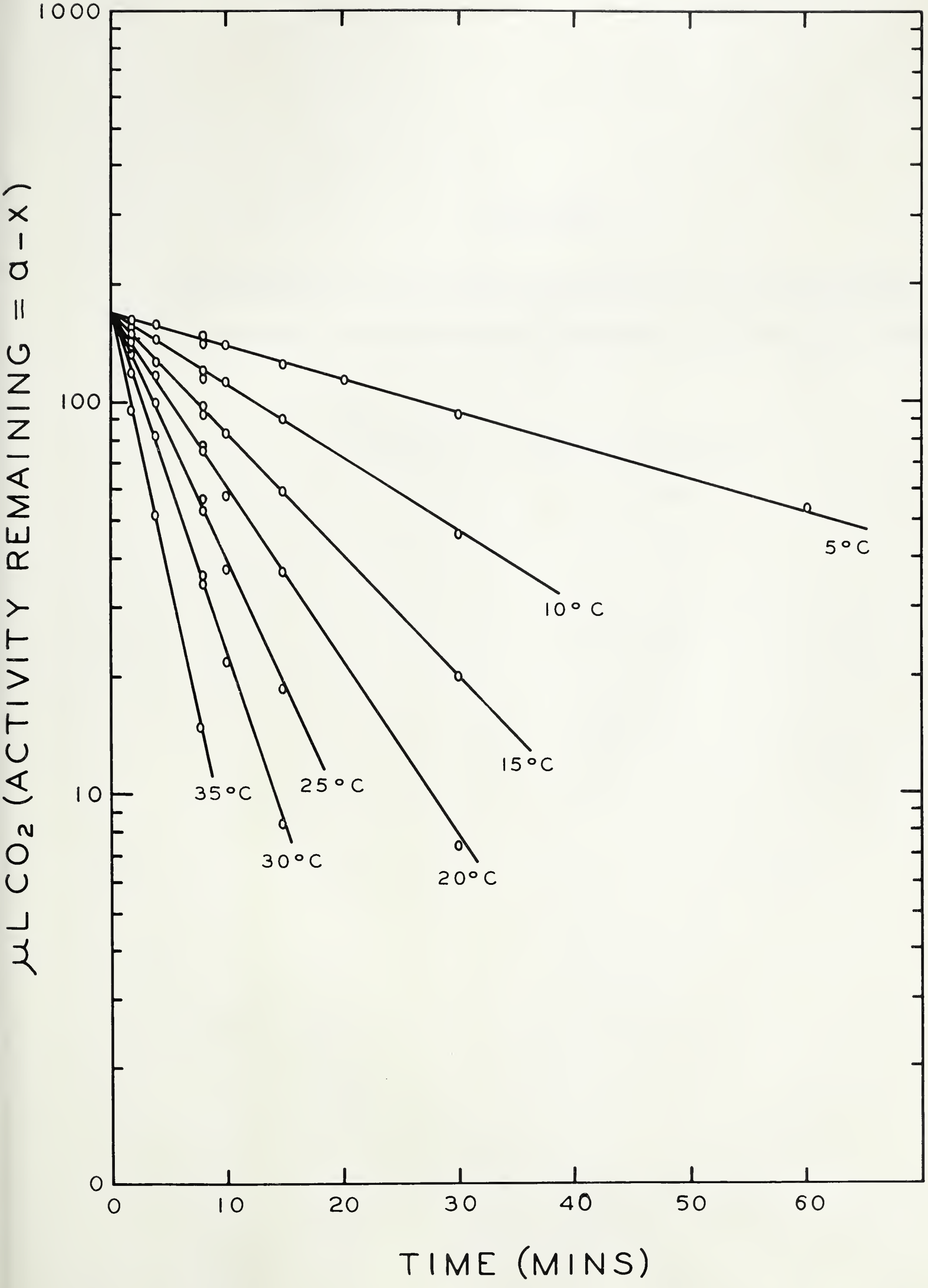

Figure 26

Effect of temperature on the rate of inhibition of non-specific cholinesterase by isosystox.

Table XVII

Pseudomonomolecular Reaction Constants (k) for the Inhibition of Non-specific Cholinesterase by Isosystox at Various Temperatures

Temperature (°C)	k (litre $mole^{-1}min^{-1}$)
5	2.61×10^2
10	4.15×10^2
15	6.67×10^2
20	9.47×10^2
25	1.33×10^3
30	1.93×10^3
35	3.03×10^3

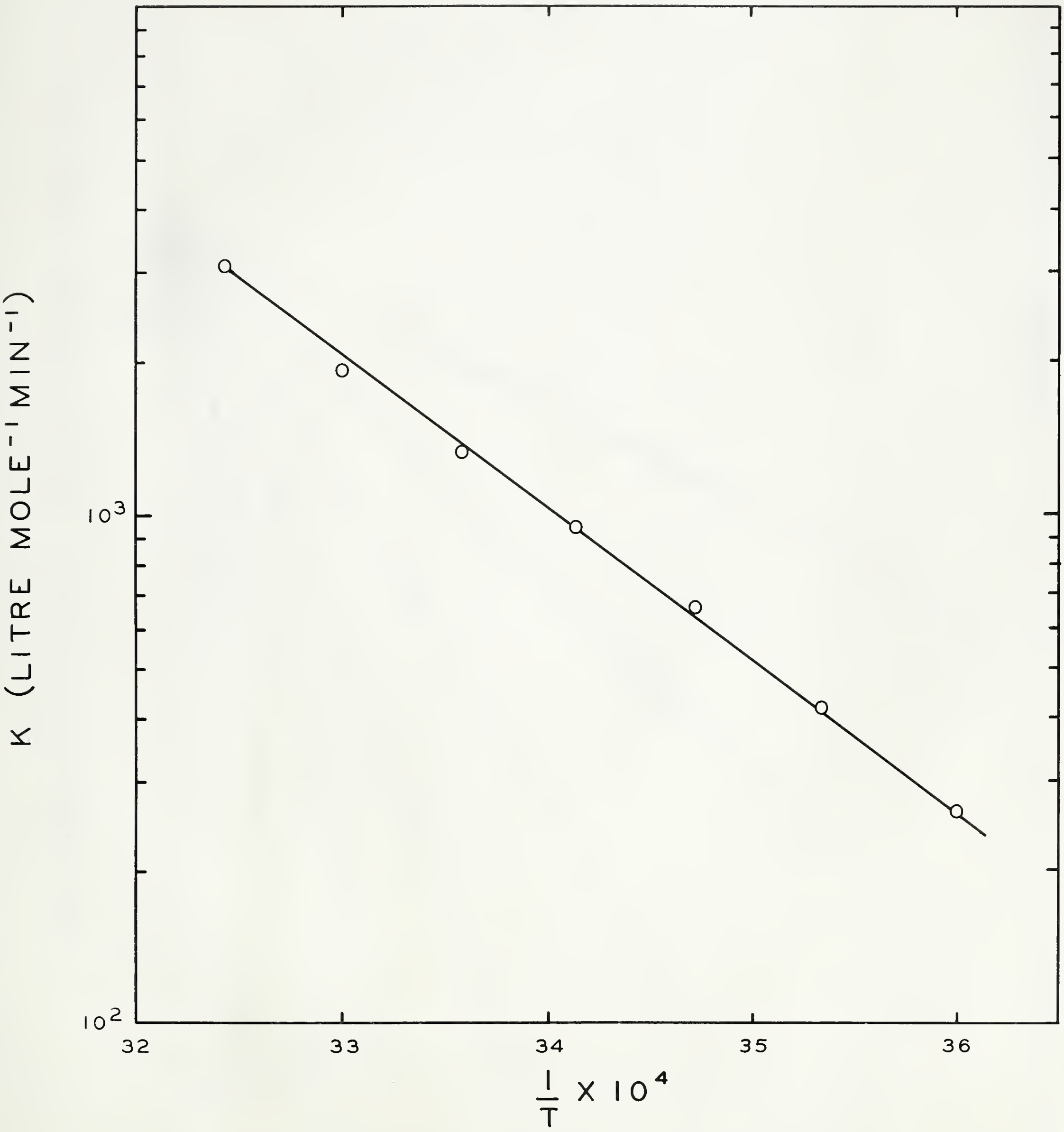

Figure 27

Effect of temperature on the rate constants for the inhibition of non-specific cholinesterase by isosystox.

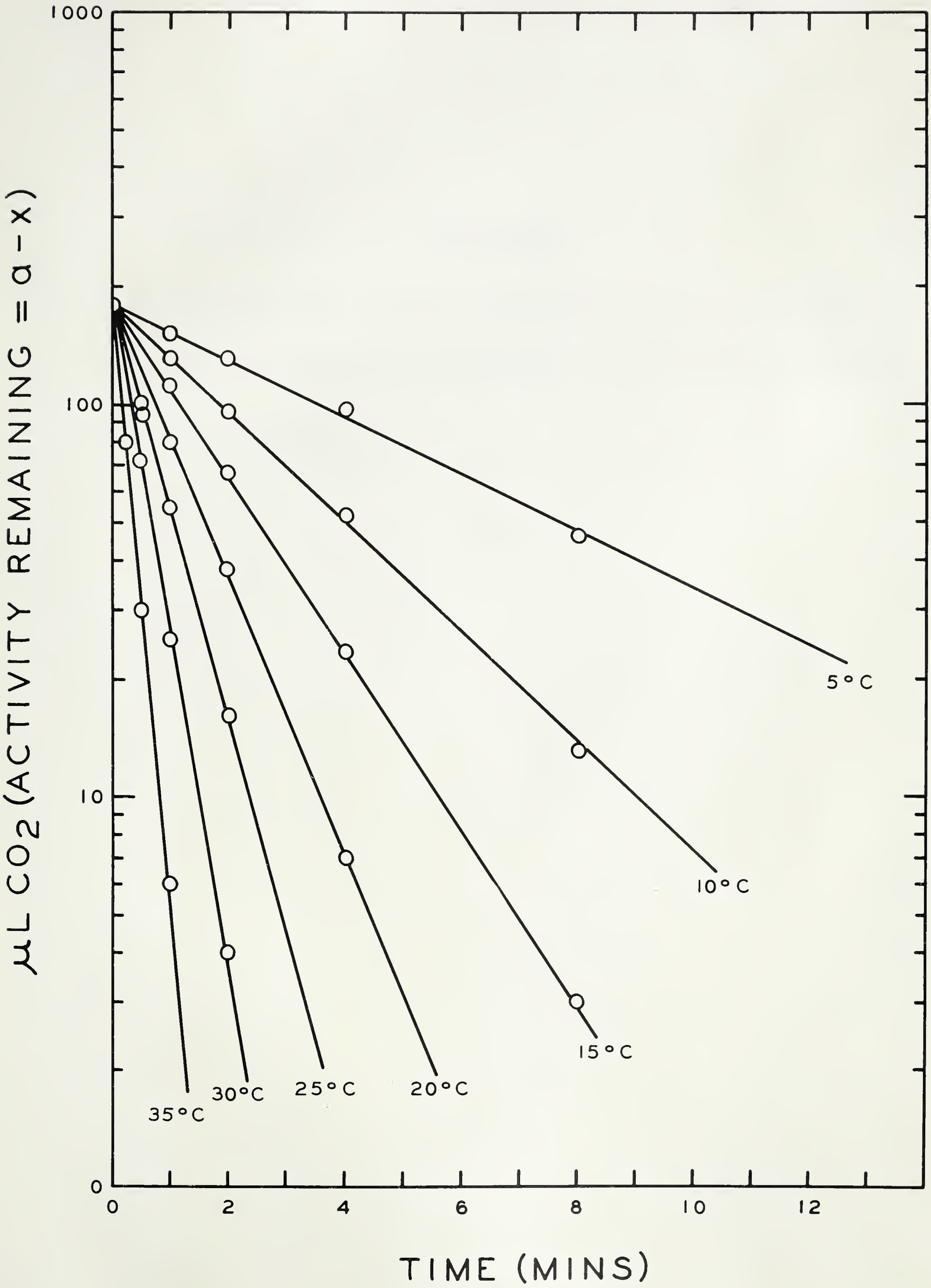

Figure 28

Effect of temperature on the rate of inhibition of specific cholinesterase by isosystox.

Table XVIII

Pseudomonomolecular Reaction Constants (k) for the Inhibition of Specific Cholinesterase by Isosystox at Various Temperatures

Temperature (°C)	k (litre $\text{mole}^{-1}\text{min}^{-1}$)
5	1.58×10^{3}
10	3.02×10^{3}
15	4.76×10^{3}
20	7.41×10^{3}
25	1.11×10^{4}
30	1.66×10^{4}
35	3.33×10^{4}

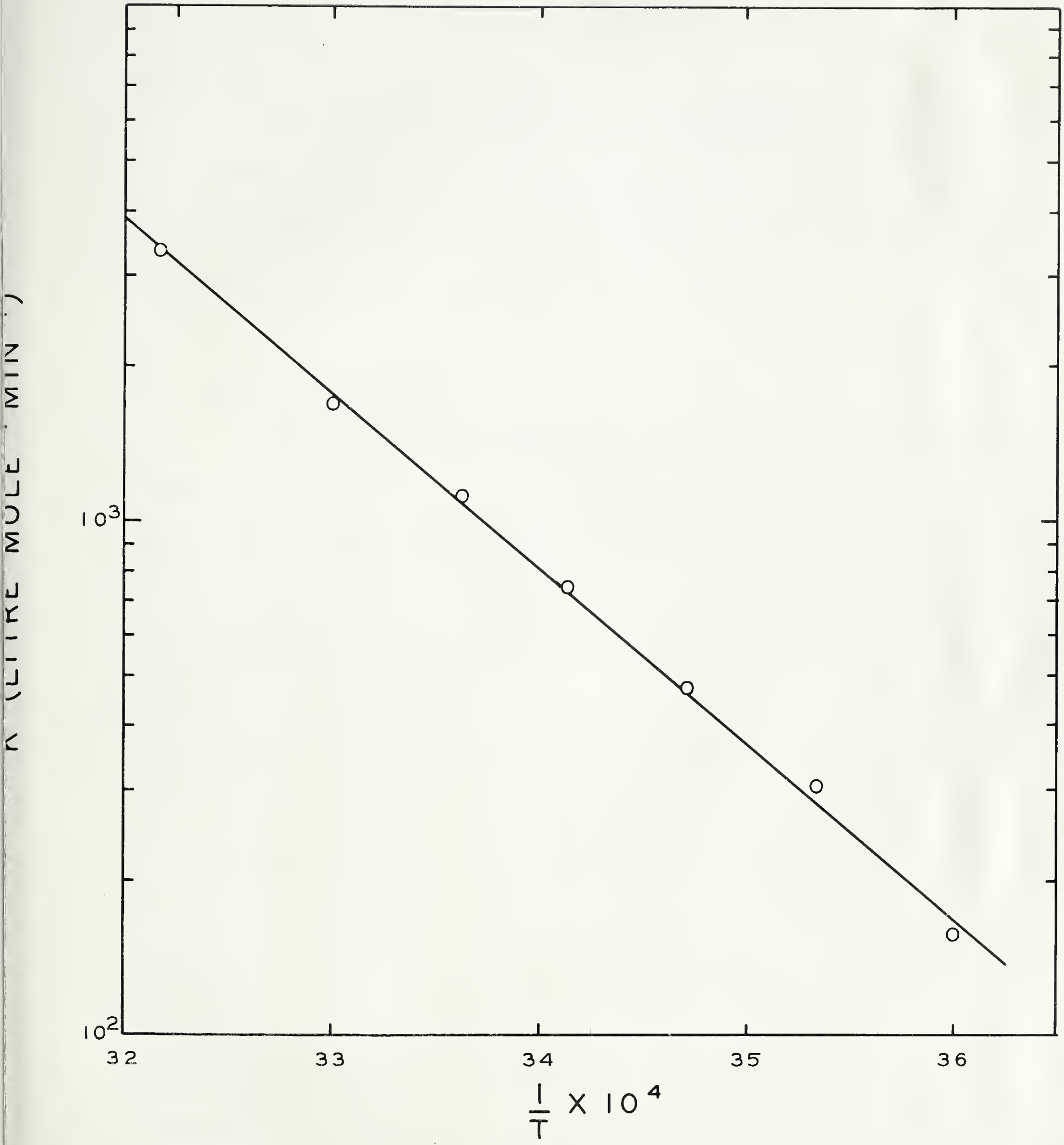

Figure 29

Effect of temperature on the rate constants for the inhibition of specific cholinesterase by isosystox.

calculated to be 15,800 cal./mole.

The activation energies for the isosystox inhibition reactions are somewhat higher than those obtained with DFP and TEPP (10-11 k cal./mole) (12), but similar to that obtained by Davison (50) for octa-isopropyl pyrophosphoramide (14.5 k cal./mole).

III General Discussion

The reaction constants for sarin and tabun show that these compounds react much more rapidly with cholinesterase than does isosystox (page 94). To evaluate the significance of this result, the time taken to inhibit the essential cholinesterase in vivo can be compared with the time taken to detoxify the injected inhibitor. The lethal dose of isosystox is approximately 8 mg./kg. in the mouse (Table XVI). At this dose the average concentration in the body would be 3×10^{-5} M isosystox.* Substituting this value for b in the equation $t_{\frac{1}{2}} = 0.69/bk$ (see page 94) and putting $k \approx 10^3$ litre mole^{-1} min^{-1}, the time for 50% inhibition of the specific cholinesterase becomes 23 minutes. If a similar calculation is made for sarin and tabun, much shorter times are obtained. The lethal dose of sarin is 0.15 mg./kg. (Table XVI) or 10^{-6} M sarin when injected intravenously in mice. Putting $k \approx 7 \times 10^7$ litre mole^{-1} min^{-1}, the time required for 50% inhibition of the specific cholinesterase becomes 0.01 minutes or less than one second. This rapid reaction allows little time for the detoxication of sarin. As noted previously (see page 36), the plasma sarinase requires nearly one minute to detoxify 50% of the sarin present. An equivalent calculation gives 0.035 minutes as the time required for 50% inhibition of the specific cholinesterase by a lethal dose

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* 4.3×10^{-5} M in body fluid only.

of tabun by the hydrolyzing enzyme.

The above calculations on the hydrolysis of sarin and tabun are based on in vitro reactions with dilute enzyme. Strauss and Goldstein (159) and Goldstein (78) have shown that there are three zones of behaviour for enzyme reactions. The Michaelis equation applies only in zone A where the enzyme concentration $[E]$ is less than 0.1 times the Michaelis constant (K_m). The zone in which the in vivo hydrolysis of sarin and tabun takes place can be calculated as follows:

Plasma contains approximately 7 g. of protein per 100 ml. If one assumes this protein to be all sarinase with a molecular weight of 10,000, for example, then the molar concentration would be 7×10^{-3}. However the purified enzyme has an activity 1200 times that of plasma (page 48). Therefore the concentration in the original plasma could not be greater than 6×10^{-6} M sarinase. This concentration would be still lower if the molecular weight were greater than 10,000 or if the enzyme could be further purified. Since the value of K_m is 7×10^{-3} M sarin (page 94) the ratio $[E] / K_m$ cannot be greater than 0.001 in undiluted plasma. The in vivo conditions for the enzymic hydrolysis of sarin (and tabun) are therefore zone A and the calculations made above regarding the time required for 50% detoxication of sarin and tabun should be valid.

The hydrolysis of the agent in the liver accounts for the difference between the intraperitoneal and intravenous toxicities of sarin and tabun. However the lethal dose of isosystox injected by the intraperitoneal route is little different from the lethal dose by intravenous injection. This is to be expected when detoxication in the liver is slow.

Isosystox is an example of a cholinesterase inhibitor which is relatively stable in the body. Although detoxication by oxidation and subsequent hydrolysis does occur (122), this process was found to be relatively slow. Sarin and tabun, as noted above, are detoxified extremely rapidly by enzymic hydrolysis in the body. Nevertheless these compounds, after intravenous injection in the mouse (Table XVI), are roughly 40 times more toxic than isosystox. This result presents a striking anomaly which requires further explanation.

The above calculations show that lethal doses of sarin and tabun should inhibit cholinesterase 1000 times more rapidly than a lethal dose of isosystox. This should result in a difference in the time taken to kill an animal after intravenous injection, as is, in fact, the case. Injection of sarin and tabun kills animals within one to two minutes whereas the toxic effect of isosystox is not apparent until about thirty minutes after injection.

Difference in inhibition rate, however, is not in itself sufficient explanation for the difference in toxicity. If all the isosystox remained unchanged in the body, the total amount required to inhibit the essential cholinesterase should not be greater than the amounts of sarin and tabun required. In other words the lethal doses of sarin, tabun and isosystox should all be approximately the same on a molar basis even though the time required to kill animals would be different. However, experimental evidence shows that the lethal doses differ greatly (Table XVI). The amount of isosystox lost by excretion or by oxidation and subsequent hydrolysis is not great enough to explain this difference in

toxicity. One other explanation for the difference could be the loss of isosystox by combination with non-specific cholinesterase and other esterases in the tissues. For example, Table XIII shows that nearly all the isosystox in 0.5 ml. of a 1.76×10^{-5} M solution i.e. 3 µg. is combined with the esterases in 0.5 g. of liver. If a similar reaction occurred in the whole body, 6 mg./kg. could be accounted for this way. This figure compares favourably with the lethal dose of 8 mg./kg. Since a large number of esterases occur in all tissues (15), the low toxicity of isosystox could be explained by the fact that large amounts combine with these enzymes.

When methods of therapy are considered, the amount of time available for a therapeutic agent to destroy unchanged anticholinesterase becomes so short as to be negligible when inhibitors such as sarin and tabun are in question. The one therapeutic mode of action (other than by drugs such as atropine which make the synapses and effector organs less sensitive to acetylcholine) is by reactivation of inhibited cholinesterase (94). Prophylaxis will be difficult since this involves destruction of the inhibitor by the anti-inhibitor at a faster rate than the inhibitor reacts with cholinesterase; this means a reaction constant greater than 7×10^{7} litre $\text{mole}^{-1}\text{min}^{-1}$.

With compounds such as isosystox, prophylaxis should be simpler since the inhibition of cholinesterase proceeds slowly. Quickly applied therapeutic measures with compounds which would destroy the inhibitor might also be effective in this case. In other words, a method which might be effective only prophylactically with sarin and tabun could be effective when applied after exposure to isosystox and similar compounds.

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